THE SITES OF ALKALINE PHOSPHATASE IN DENTINOGENESIS [1]

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EIGHT FIGURES

INTRODUCTION

The enzyme alkaline phosphatase has been the subject of considerable investigation since its relation to the formation of calcified tissues was first postulated. Alkaline phosphatase was known to be present in osseous tissues. However, it was not until recent years, when adequate methods were perfected, that the histochemical localization of this enzyme has met with much success.

Because of the nature of the material, only limited work has been done on the localization of the enzyme in dental tissues, and its exact location within the dental pulp is controversial. It was the purpose of this investigation to attempt to contribute further information on this subject.

HISTORICAL

Robison and Soames ('24) proposed the hypothesis that calcium phosphate present in bone was in equilibrium with a saturated solution of calcium phosphate in the plasma. The enzyme, alkaline phosphatase, present at the sites of calcification aided in the hydrolysis of plasma hexosemonophos-

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phate esters, releasing phosphate ions. The solubility product of calcium phosphate was thus exceeded, and a precipitation of calcium phosphate resulted.

This theory has generally been accepted. However, Roche and Mourgue ('42a, b) investigating the calcium and phosphorus content of various embryonic bones and developing sharks' teeth, found that during bone development the amount of phosphorus present was in excess of the amount needed to precipitate the calcium present. They concluded, therefore, that alkaline phosphatase was probably not directly involved in the final calcification of bone. Rather, it aided in the initial accumulation of phosphate ions which were independently fixed. It was after this initial stage that actual calcification occurred.

Although the presence of alkaline phosphatase in various tissues was verified by many workers, it was not until 1939 that adequate methods were devised for the histochemical localization of the enzyme. In that year, both Gomori ('39) and Takamatsu ('39) published methods for its visualization.

Kabat and Furth ('41) and Engel and Furuta ('42) were among the first to apply the Gomori technique to the localization of alkaline phosphatase in dental tissues. The latter, using incisors and molars of 1- to 4-day-old rats, found indications of high alkaline phosphatase activity in the dental pulp subjacent to odontoblasts underlying calcifying dentin. There was no phosphatase activity in the basal portion of the tooth where odontogenic epithelium was undergoing differentiation. It was found that the odontoblastic layers showed no phosphatase activity. However, in certain cases a slight positive reaction was obtained, but this was assumed to be an artifact.

Horowitz ('42) investigated the location of alkaline phosphatase and glycogen in the heads of embryonic rats. He found both glycogen and phosphatase in the dental pulp near the sites of active proliferation. He also found the enzyme in the incisal portion of the developing tooth.

Gomori ('43) developed another technique for the histochemical demonstration of alkaline phosphatase. Upon examination of rat embryos, he found high alkaline phosphatase activity in the connective tissue of the pulp, particularly subjacent to those regions in which calcified dentin was present. He found the odontoblasts to be negative with only occasional traces of activity.

Using the Gomori technique on the teeth of embryonic pigs, Bevelander and Johnson ('43) found alkaline phosphatase highly concentrated in the pulp and combined epithelium adjacent to the odontoblasts just prior to and during calcification. They also found the odontoblasts, themselves, and the odontoblastic processes strongly positive. The authors suggested that the function of the odontoblast was the mediation of enzyme transfer.

The following year Bevelander and Johnson ('46) repeated their experiments. This time, however, they included ground sections of calcified teeth of older embryos. Their results were similar to those found previously.

Morse and Greep ('47) attempted to determine the effect of pH on phosphatase activity and reported the possible presence of two enzymes: a nuclear phosphatase which was demonstrable from pH 7.0 to 9.0, and a cytoplasmic phosphatase which was evident between pH 9.5 and 11.5.

They found the cytoplasm of the odontoblast to be positive with the most active sites adjacent to active calcification. No phosphatase was noted in the areas of greatest proliferation.

Greep, Fischer, and Morse ('48) examined the dental tissues of 4-day-old and 28-day-old rats subjected to various decalcifying solutions. Their findings, with regard to the localization of alkaline phosphatase were similar to those found by Morse and Greep. They did, however, report a darkening of the predentin border, but described it as an artifact since they found a similar darkening in their controls.

Bruckner ('49) reported on the effect of decalcification on the alkaline phosphatase activity on the teeth of a human

term fetus. He noted that the decalcified sections showed varying degrees of enzyme inactivation, the precise localization of the enzyme being altered. He also reported that, in the undecalcified tooth, alkaline phosphatase appeared to be distributed throughout the intercellular substance, in the odontoblasts, and in the walls of the blood vessels.

Bevelander and Johnson ('49) again repeated their experiment on the dental tissues of embryonic pigs. Their results were similar to those reported previously. However, pH studies failed to reveal the presence of two phosphatases as reported by Morse and Greep.

MATERIALS AND METHODS

For this study two series of Wistar strain rats were used. The first series of rats were one- to three-day-old litter mates. These animals were decapitated; and the heads were split mid-sagittally and fixed in 80% alcohol for 7 days. The undecalcified heads were then dehydrated in dioxane and embedded in paraffin. The blocks were cut at tissue thicknesses of 10 μ and the sections mounted on slides by water floatation. The sites of alkaline phosphatase activity were visualized by modifying the Gomori cobalt sulfide technique.

The Gomori cobalt sulfide technique consisted of incubating sections of tissue at 37°C. for one and one-half hours in a solution containing:

Sodium β glycerophosphate	0.46%
Sodium barbital	0.46%
Calcium chloride	0.16%

Following incubation, the sections were washed in 1% calcium chloride solution; after which they were placed in 2% cobaltous chloride for two minutes, washed in distilled water, and then placed in 1% ammonium sulfide for one minute. The sections were counterstained with hematoxylin and triosin. The modification of this procedure consisted in varying the times of incubation from 0 to 90 minutes. The sections were coated with 1% celloidin just prior to incubation. They were treated only with absolute alcohol after paraffin removal.

The second series of rats were 4 hours old. They were decapitated; the mandibles split, cut at the ramus, skinned, and dropped into isopentane cooled to the temperature of liquid air. The tissues were dried in a cryostat under 1 mm of mercury pressure at a temperature of — 78°C. for 72 hours. Upon removal from the cryostat the tissues were immediately placed in xylene. They were then embedded in paraffin and sectioned at 10 μ. The sections were floated on slides with two drops of water per slide and spread on a warm (40°C.) plate. Excess water was immediately removed by blotting. Immediately prior to incubation all sections were treated with absolute ethyl alcohol for one minute to fix the soluble proteins and then coated with 1% celloidin. The sites of alkaline phosphatase activity were visualized by a modified Gomori technique and counterstained with hematoxylin and triosin. Incubation times were varied in this series.

In addition to varying the incubation time, the following changes in the technique were made. Following incubation, the tissues were immediately placed in a 1% calcium chloride solution at 4°C. to stop enzymatic action. They were then washed in 1% calcium chloride until the wash solution no longer gave a test for barbital. It was found that 6 one-minute washings were adequate. It was also found that 6 one-minute washings in distilled water, following the cobaltous chloride replacement, were sufficient to remove all traces of free cobalt ions.

Control sections from both series were treated with the above techniques except that sodium β glycerophosphate was omitted from the incubation mixture.

Several sections of the second series were incubated omitting the replacement of calcium phosphate by cobalt sulfide. The sections were then observed under the polarizing microscope.

The localization of alkaline phosphatase in the incisors was studied.

RESULTS

The first series of slides showed a diffuse precipitate of cobalt sulfide in the dental pulp adjacent to those areas of active calcification. It was interesting to note that by the first postnatal day active calcification had proceeded almost to the basal portion of the incisor and that alkaline phosphatase was to be found in the pulpal tissue subjacent to the odontoblasts in that area (fig. 1).

Localization of the enzyme with respect to the odontoblast could not be determined satisfactorily, since such a diffuse heavy deposition was present that it could not be determined whether the enzyme was intracellular or extracellular.

Also noted was a dark line at the predentin-pulpal border. This line was not observable in the control sections (fig. 2).

The sections obtained by the frozen-dried method presented a more detailed picture. It was found that at an incubation time of 10 minutes activity began to appear in the pulp, extracellularly, closely associated with the intercellular substance (figs. 3 and 4). The enzyme was visible also in the odontoblastic layer. It was found in the cells as a light brown precipitate and extracellularly as fine reticular lines below and between the odontoblasts.

In sections incubated from 20 to 50 minutes the enzyme appeared to have its greatest concentration in the pulp subjacent to the odontoblasts in the incisal portion of the tooth (fig. 5). The sites of activity showed increasing color intensity as the time of incubation was prolonged. Dark lines indicated activity between the odontoblasts, and a light brown color indicated slight activity within the odontoblasts themselves. The dark lines between the odontoblasts crossed the odontoblastic-predentinal border and continued upward into the predentin (fig. 6). There was also noted an extracellular concentration of enzyme associated with the collagenous tissues of the pulp surrounding the blood vessels of the pulp (fig. 7). No apparent enzyme activity was found associated with the odontoblastic processes.

It was also found that the cobalt sulfide deposition in this series of tissues appeared well defined at the predentin-pulpal border, rather than diffuse as noted in those sections of the first series.

As the incubation time was prolonged, it was noted that although the over-all picture of enzyme location did not change, the deposition of cobalt sulfide became more dense, and the cellular picture was obscured. Sections incubated more than 60 minutes were difficult to interpret due to the heavy deposition of the salt as seen in figure 8.

The calcium phosphate precipitated during incubation was not detectable in those sections examined with the polarizing microscope. Only preformed dentin was observed.

DISCUSSION

The controversy that currently exists regarding the localization of alkaline phosphatase within the calcifying tooth may be the result of several sources of error inherent in the technique used. The first would appear to be the use of decalcifying fluids. As was shown by Bruckner ('49), their use on tissues results in inactivation of alkaline phosphatase and an apparent change in position of the enzyme. It must be noted that decalcification below pH 4.8 results in irreversible inactivation of the enzyme, and decalcification of the dense oral tissues at any pH above this means exposing the enzyme to unfavorable conditions for long periods of time.

In any histological or histochemical study, methods of fixation are of prime importance since improper fixation results in a loss of cellular relationships. This is very important in the study of dental tissues since the soft pulp is encased in a hard calcified shell. Any shrinkage which may result from alcohol fixation results in the pulp drawing away from the dentin. Since the tearing at the pulpal-dental border may be irregular, it may result in distortion with the loss of proper cellular relationships.

Examination of sections fixed in alcohol showed that at some time during the procedure there was a diffusion of enzyme from the original sites of enzyme activity. This was not found in the frozen-dried tissues. The actual phase of the technique during which this diffusion took place was not determined. It would appear, however, to be either the result of the alcohol fixation, or of floating the tissues onto slides from a water bath. This conjecture was supported by the fact that both series of tissues were treated identically from that point on. Yet, the frozen dried tissues did not show evidence of enzyme diffusion.

Ruyter and Neumann ('49) found a similar diffusion when localizing alkaline phosphatase in kidney fixed in 80% alcohol. They believed it to be due to the solubility of the enzyme in the low concentration alcohol solutions used after removing paraffin from the sections.

In the studies discussed here, however, such alcohol treatment was avoided, but diffusion was still noted in alcohol fixed tissues.

Another source of error noted in these experiments was the retention of barbital buffer by the tissues following incubation. If the tissues were not adequately washed before being treated with cobalt chloride, a diffuse precipitate of barbiturate resulted, leading to a false picture of alkaline phosphatase activity.

Before attempting to obtain interpretable results, attempts were made to reduce these sources of errors to a minimum. To avoid inactivation of the enzyme by decalcification, the animals used were not old enough to require decalcification. The frozen-dried technique was used to avoid the errors inherent in alcohol fixation. The resulting sections showed greater enzyme activity with minimum tissue shrinkage. It was found, however, that the cellular picture was lost due to the solubility of certain of the proteins. To overcome this the sections were subjected to treatment with absolute ethyl

alcohol for one minute to denature these proteins. The solubility of the proteins was lost with no noticeable loss of enzyme activity.

Nothing can be said here about the possible diffusion of enzyme resulting from mounting the tissues from a water bath. The individual mounting of sections with the immediate removal of excess water was used as a routine precautionary measure.

Time studies were used in order to demonstrate the sites of greatest enzyme activity. Previous investigators had incubated their tissue sections for long periods of time to bring out maximum activity as denoted by the heavy cobalt sulfide precipitation on their sections. This, in itself, may lead to a false picture.

The enzyme is but slowly inactivated during the progress of the reaction it catalyzes. Long periods of incubation, then, do not bring out maximum activity. Rather, there is a deposition of calcium phosphate which eventually becomes so dense that no further activity can be observed upon prolonging the incubation period. The actual sites of activity are therefore obscured by the dense precipitate.

In pursuing this investigation, it was felt that the determination of areas of greatest activity was more important than obtaining the heaviest precipitation possible. In time studies those areas which have the greatest activity are those which appear first.

Morse and Greep ('47); Greep, Fischer, and Morse ('47); and Bevelander and Johnson ('45, '46, '49) in their studies, emphasized the importance of the intracellular enzyme with regard to calcification of dentin. Gomori ('43) and Engel and Furuta ('42), however, stressed the importance of the extracellular enzyme, having found only traces of activity in the odontoblasts. The difference in the results obtained must have been due to differences in their techniques.

In these studies, both intracellular and extracellular alkaline phosphatase were found; however, it would appear that the extracellular enzyme activity, as qualitatively determined

by the technique used, was greater than that of the intracellular enzyme. In addition, no enzymatic activity was noted in the odontoblastic processes, and only traces in the predentin. However, a high degree of activity was noted extracellularly at the predentin-pulpal border and in the pulp itself. Apparently the enzyme believed to be responsible for calcification has its greatest activity extracellularly at a position comparatively remote from the actual site of calcification. There are several hypotheses which may explain such a situation. However, before any explanation of the actual mechanism of dentin calcification can be accepted, further biochemical and histological investigation is necessary.

SUMMARY

1. A modification of the Gomori technique for the histochemical localization of alkaline phosphatase in partially calcified rat incisors has been described.

2. A comparison was made between the localization of enzyme in tissues fixed in 80% alcohol and frozen-dried tissues. Tissues prepared by the frozen-dried technique exhibited greater enzyme activity and less enzyme diffusion.

3. The greatest activity was found in the pulp subjacent to areas undergoing active calcification, and at the predentin-pulpal border. Slight activity was noted in the odontoblasts and in the predentin.

4. The problem involved in having alkaline phosphatase with its greatest activity at a position comparatively remote from that of actual calcification was cited.

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PLATE 1

EXPLANATION OF FIGURES

A., ameloblastic layer; B., bone; D., dentin; Od., odontoblastic layer; P., pulp; Pd., predentin; Ph., phosphatase activity; Po., phosphatase activity at the predentinal-pulpal border.

1 Lower central incisor of one-day-old rat; section incubated 90 minutes. Note the heavy precipitate throughout the section; particularly in the odontoblastic layer and pulp.

2 Lower central incisor of one-day-old rat. Control. Note the complete absence of phosphatase activity. Alcohol fixed and counterstained with haematoxylin and triosin after incubation. × 65.

3 Incisal portion of lower central incisor of 4-hour-old rat; section incubated 10 minutes. Note the phosphatase activity in the pulp below the odontoblasts, and the reticular lines projecting into the odontoblastic layer. Frozen-dried. × 100.

4 Higher power of figure 3. × 400.

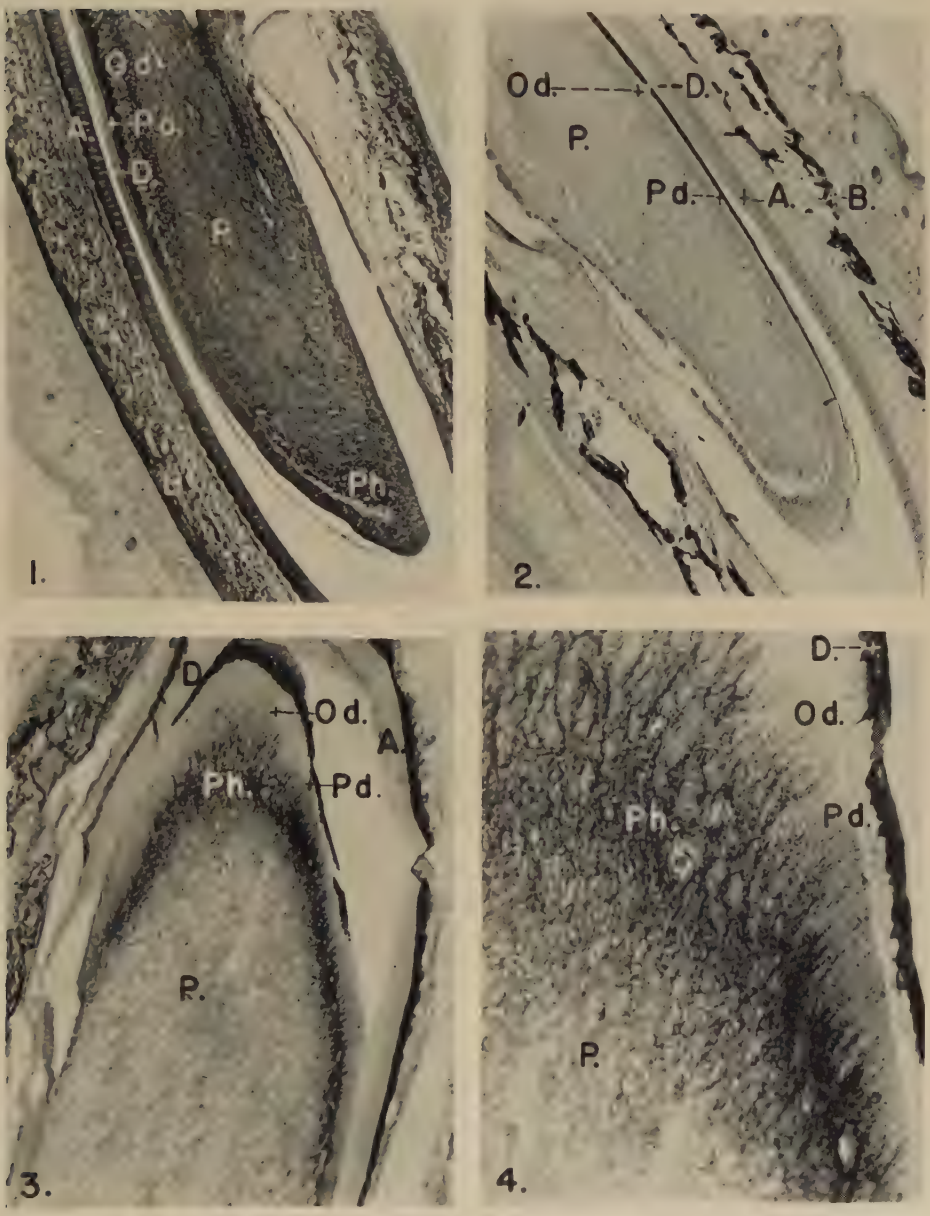
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PLATE 2

EXPLANATION OF FIGURES

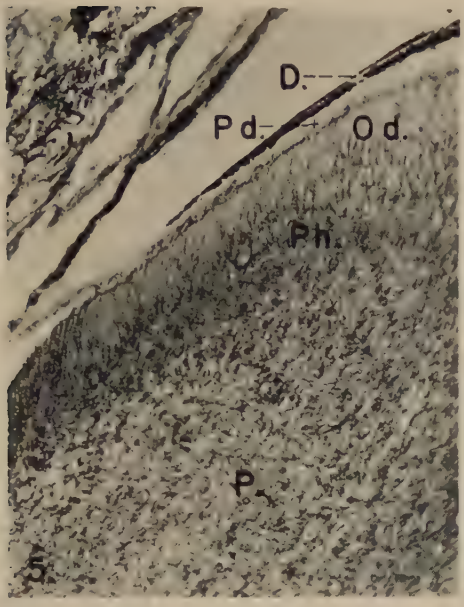
C., capillary; D., dentin; Od., odontoblastic layer; P., pulp; Pd., predentin; Ph., phosphatase activity.

5 Incisal portion of lower central incisor of 4-hour-old rat; section incubated 40 minutes. Note the heavier deposition resulting from phosphatase activity. The reticular lines completely traverse the odontoblastic layer. Frozen-dried. × 200.

6 Higher power of figure 5. Note that the reticular lines continue across the odontoblastic-predentinal border and appear to continue into the predentin. Frozen-dried. × 400.

7 Pulp of lower central incisor; section incubated 40 minutes. Note the increased phosphatase activity around the capillaries of the pulp. Frozen-dried. × 200.

8 Incisal portion of lower incisor of 4-hour-old rat; section incubated 90 minutes. Note the general loss of detail due to over-precipitation resulting from prolonged incubation. Frozen-dried. × 200.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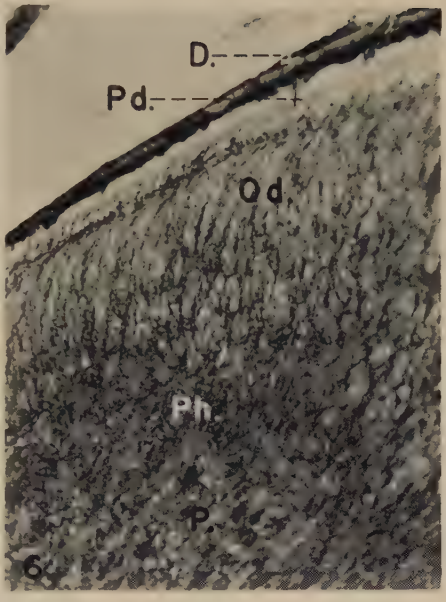
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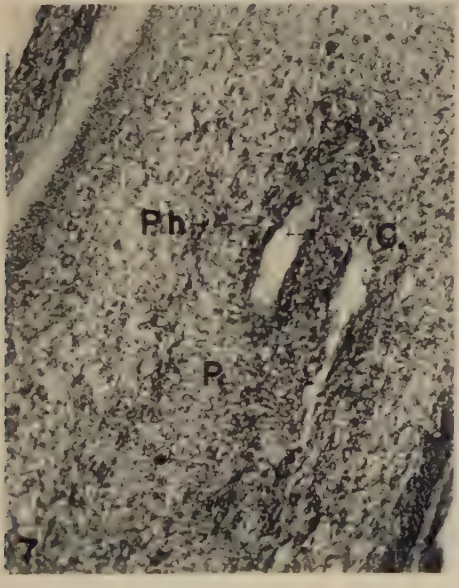
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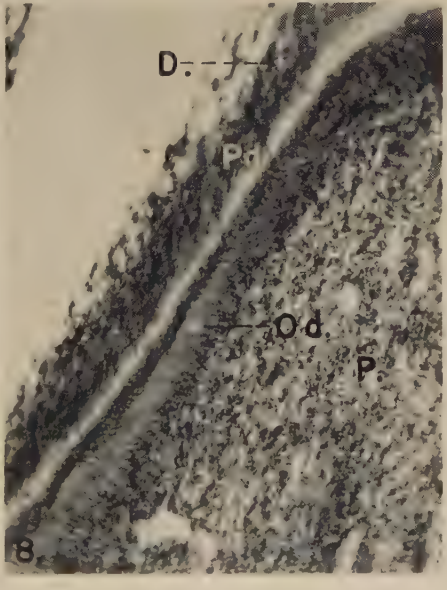
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CHANGES IN ALKALINE PHOSPHATASE ACTIVITY OF RAT VAGINAL EPITHELIUM DURING THE ESTROUS CYCLE

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TWELVE FIGURES

Increase in alkaline phosphatase activity of vaginal epithelium of the mouse following administration of estrogen to spayed animals has been reported by Jeener ('47) and Kamell and Atkinson ('48). The present study represents an attempt to correlate the changes in localization and intensity of phosphatase activity in vaginal epithelium with the structural changes occurring during the growth and regression of this membrane seen in the several stages of the estrous cycle of the normal rat.

MATERIAL AND METHODS

A large group of albino female rats was examined by daily vaginal smears through 3 or 4 estrous cycles. The smears were obtained by lavage in order to minimize trauma to the vagina. Eighteen animals which had exhibited normal cycles were sacrificed, 5 each in diestrus, proestrus, and estrus and three in metestrus.

Five female rats spayed for periods of from 2 to 5 weeks were injected with estradiol benzoate in oil. The total amounts administered over three days ranged from 0.15 to 0.5 mg. Five uninjected spayed females served as controls. The spayed animals were killed on the day following the last injection.

All animals were killed by decapitation, and a middle segment of vagina was removed and fixed in cold 80% alcohol. Tissues were sectioned in paraffin at 7 μ and alternate sections stained by the alkaline phosphatase technique of Gomori ('41) (modified by the addition of 0.1 M $MgSO_4$ to the incubating medium) or with Harris' hematoxylin and eosin. The alkaline phosphatase preparations were incubated in glycerophosphate at pH 9.4 and 37°C. for periods of one-half, one, two, and twenty-four hours.

RESULTS

Diestrus. As indicated by the observed deposits of cobalt sulfide, alkaline phosphatase activity in the vaginal epithelium of the normal diestrous rat grades from moderately strong at the free surface to weak or negligible in the region of the basement membrane (fig. 1). The area of highest activity of this enzyme appears to coincide with the narrow superficial band of parakeratosis seen in routine histologic preparations. The precipitate is uniformly distributed in the surface zone, but it is concentrated at the periphery of cells and in their nuclei in the intermediate zone. Cobalt sulfide is faintly visible only in the nuclei in the basal layers.

Evidence of marked phosphatase activity is seen in blood vessels of the superficial stroma. Deeper vessels are not visible in phosphatase preparations, except for a few, isolated or in small groups, which suggest a moderate enzymatic activity. No change is observed in the apparent distribution or activity of alkaline phosphatase in stromal vessels during the estrous cycle.

Proestrus. In the early hours of the proestrum the vaginal epithelium of the rat increases considerably in thickness. The cells of the superficial zone appear hydropic in routine histologic preparations. This layer which represents the surviving epithelium of the previous cycle is separated from the deeper strata by a thin band of cornified cells and a layer of granular cells. The surface cells desquamate producing the cellular detritus characteristic of the proestrous vaginal

smear. Thus in later proestrus the vaginal epithelium has a thin layer of keratinized tissue at the surface resting on a layer of granular cells.

In sections prepared by the Gomori method, intense blackening indicative of high phosphatase activity occurs in the superficial zone and in the cells deep to the cornified layer but not in the cornified or basal layers (fig. 2). Later in proestrus (fig. 3) the precipitate appears in the superficial cornified zone, and still later (fig. 4) it is present to within one or two layers of the basement membrane.

Preparations incubated for one-half to two hours exhibit, in that zone of the stratum germinativum which shows evidence of high phosphatase activity, a concentration of precipitated cobalt sulfide at the periphery of cells and in the intercellular spaces. With prolonged incubation (24 hours) the precipitate is more uniformly distributed through the cells, and the intercellular spaces become visible as such (fig. 5).

The superficial cells of early proestrus stain intensely for phosphatase. The precipitate is found in the cytoplasm and in the vicinity of the cell and nuclear membranes, where it is more concentrated. Other foci of apparently high activity of this enzyme are the granules of the granulosa layer and coarser granules located in the inner portion of the cornified zone. These latter granules contain some pre-formed phosphate as evidenced by their slight staining in control slides incubated without substrate.

In the vaginal epithelium of some of the proestrous animals precipitate occurs in certain regions only in isolated cells or groups of cells (fig. 6). This seeming discontinuity in the activity of the enzyme may represent a functional difference between contiguous segments of the membrane or it may be an artefact. The presence of a heavy precipitate in blood vessels adjacent to unstained areas of epithelium would appear to militate against the latter view.

Estrus. The distribution of cobalt sulfide in the outer layers of the germinative zone and, less concentrated, in the desquamating cornified layer suggests that the zone of phosphatase activity has moved toward the surface in the vaginal epithelium of the estrous animal (fig. 7). Most areas show the deeper one-half or more of the non-keratinized portion of the epithelium to be devoid of precipitate except for a trace in nuclei of some deep lying cells.

In the vaginal epithelium of one estrous rat the precipitate is not present in a continuous layer but has an irregular distribution (fig. 8). In this tissue scattered groups of cells showing evidence of moderate to high phosphatase activity are seen even in the deepest layers of the epithelium.

Metestrus. Precipitated cobalt sulfide is present in all layers of the metestrus vaginal epithelium but is more concentrated at the free surface than at the base of the epithelium (fig. 9). The intensity of the coloration is less than at any other stage of the estrous cycle, and the deposit does not concentrate in the intercellular areas as it does in the other stages.

Spayed. Female rats bilaterally ovariectomized for periods up to 5 weeks still show evidence of some phosphatase activity in a narrow band at the free surface (fig. 10). In some areas the epithelium is only one or two cell layers in thickness, but even here evidence of phosphatase activity remains. In the vagina of one animal adjacent to areas exhibiting marked castration change there is a zone of apparent mucification of the surface cells. These cells give evidence of marked phosphatase activity (fig. 11).

Vaginal epithelium of spayed animals receiving estrogen injections resembles generally that of normal estrous females. Numerous mitotic figures, hyperplastic epithelial pegs and a wide cornified band afford evidence of a conspicuous growth activity. Cobalt sulfide is precipitated in intensely black deposits which extend more deeply into the basal layers than in the normal estrous vagina (fig. 12).

DISCUSSION

It can be concluded from the observations reported above that one of the results of administration or cyclic release of estrogenic hormone is the mobilization or activation of alkaline phosphatase in vaginal epithelium of the rat. It has been suggested that the endocrine regulation of enzyme systems may be related to the mechanism of action of certain hormones (Dempsey, Greep and Deane, '49).

The principal histologic effect on vaginal epithelium of the pre-estrual increase in circulating estrogen in the normal female rat is the hyperplastic growth of this membrane and its ultimate cornification. The apparent distribution of alkaline phosphatase in the vaginal epithelium during its active growth phase suggests that the enzyme is largely concerned with keratin formation. Thus, during proestrus and, more conspicuously, in estrus, only moderate enzyme activity is indicated in the germinative zone, whereas highest activity is associated with those cells of the intermediate layers which are becoming keratinized. Kamell and Atkinson ('48) report a somewhat similar distribution of alkaline phosphatase in the vagina of the spayed, estrogen-treated mouse, with a parallel gradient in the concentration of cytoplasmic ribonucleic acid. The growing evidence in support of the idea of a relationship between alkaline phosphatase and pentose nucleic acid in the biosynthesis of proteins has been discussed by Moog ('46).

The histological similarity between vaginal mucosa and skin is compelling, and it is noteworthy that somewhat similar histochemical results have been reported for rapidly growing skin. Biesele and Biesele ('44) describe a strong phosphatase reaction in the deeper layers of epidermis of mice painted with methylcholanthrene. Fell and Danielli ('43) find alkaline phosphatase activity greatly increased in (rat) skin healing from experimental wounds and burns. In both cases it is apparent that phosphatase activity is highest in the superficial dermis and is relatively low in epidermis as compared with vaginal epithelium; however, normal skin ex-

hibits a negligible phosphatase activity. It may thus be postulated that maintenance of the cornified surface against normal attrition is a sufficiently slow process that the requisite enzyme concentration or activity is less than that demonstrable by customary histochemical techniques.

Kamell and Atkinson ('48) found complete absence of alkaline phosphatase and mucin in the vaginal epithelium of mice 3 to 4 weeks after ovariectomy. Mucification of the surface epithelium followed injections of estrogen and progesterone. In the present investigation no spayed rat evidenced complete absence of phosphatase activity in vaginal epithelium up to 5 weeks postoperatively. In a single instance conspicuous mucification of the superficial epithelium (periodic acid leuco-fuchsin stain) was observed in the vagina of an animal ovariectomized for 5 weeks, which had received no injections and had been caged alone. Autopsy revealed no regeneration of ovarian tissue in this or any other spayed rat.

Discontinuity in the distribution of alkaline phosphatase as suggested by preparations from some of the proestrous and estrous vaginas is more marked in buccal mucosa of rats in the same stages of the estrous cycle (Ring, '49). Assuming that a relationship between phosphatase activity and keratin formation does exist and that the observed discontinuity represents a functional difference in local concentration or activity of this enzyme, it may be concluded that under certain conditions keratinization is an intermittent process, occurring at a given time in scattered areas and being suspended in adjacent areas of a continuous epithelial membrane. A morphological counterpart not infrequently observed in thick epidermis is discontinuity of the stratum granulosum and stratum lucidum.

The vastly different histological appearance in preparations of proestrous vagina incubated for one-half and 24 hours (figs. 4 and 5) suggests the necessity for caution in interpreting the localization of active phosphatase enzyme from sections incubated for long periods of time. It is ap-

parent that there may be considerable diffusion of the enzyme or the demonstrable products of its activity with prolonged incubation.

SUMMARY

1. Alkaline phosphatase preparations of the vaginal epithelium of normal albino rats suggest that the activity of this enzyme increases from a minimum during metestrus and early diestrus to a maximum during the stage of proestrus.

2. In the zones of high enzymatic activity the highest concentration of precipitated cobalt sulfide occurs at the periphery of the cell.

3. Alkaline phosphatase activity appears to be especially associated with those layers of the epithelium in which formation of keratin is taking place, viz. outer germinative and granular strata.

4. The possible relationship between alkaline phosphatase activity and keratinization is discussed.

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PLATE 1

EXPLANATION OF FIGURES

All figures are unretouched photomicrographs of rat vaginal mucosa, and, with the exception of figure 11, all preparations are stained by the alkaline phosphatase method of Gomori.

1 Second day of diestrus. Incubated one hour. × 200.

2 Early proestrus. Incubated one-half hour. × 250.

3 Same animal as figure 2, but adjacent region showing membrane immediately after superficial layer desquamates. Incubated one-half hour. × 250.

4 Late proestrus. Incubated one-half hour. × 500.

5 Same tissue as figure 4. Incubated 24 hours. Note apparent change in distribution of enzyme. × 500.

6 Early proestrus showing discontinuous zones of precipitation. Incubated one hour. × 250.

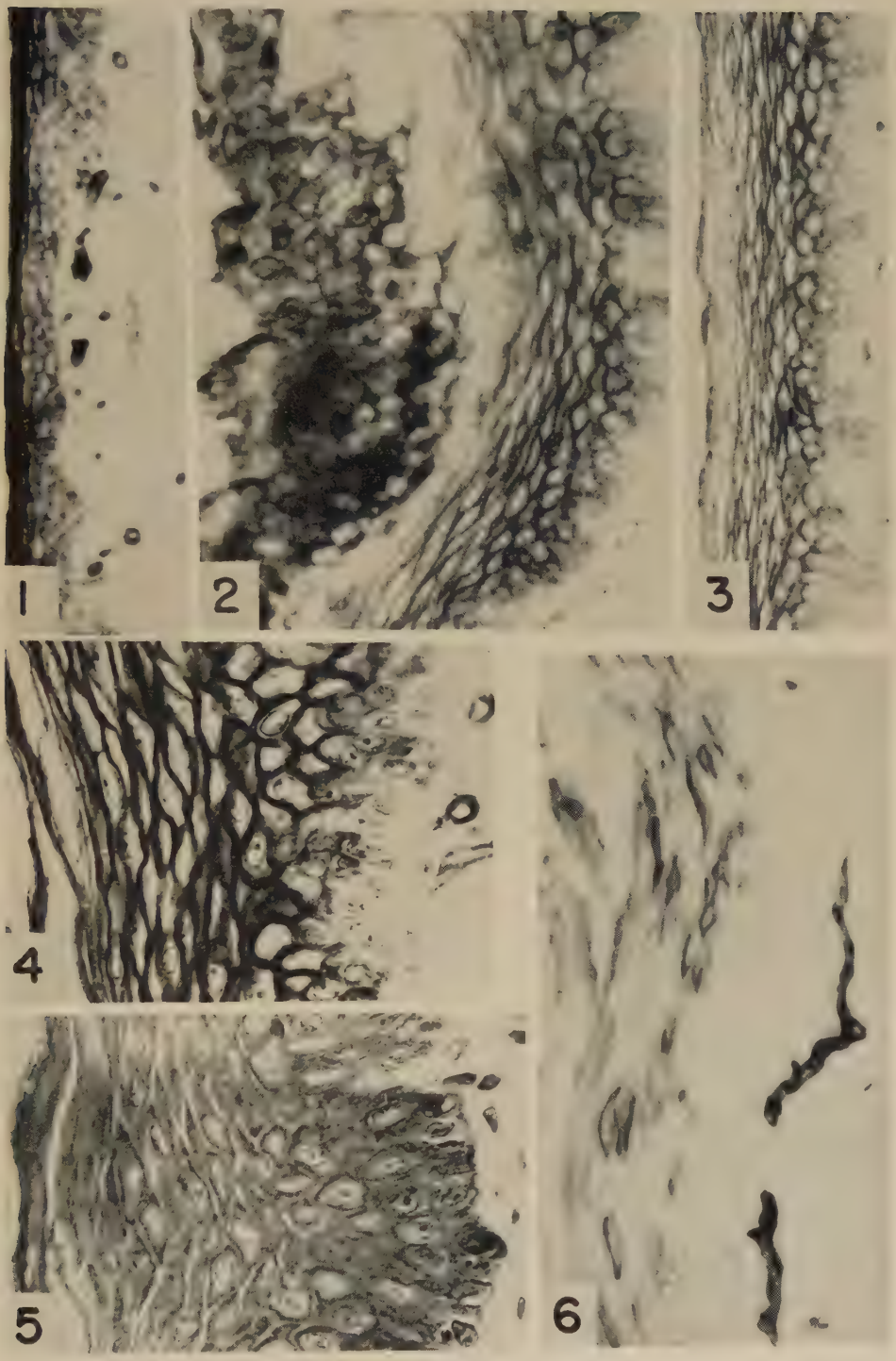
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PLATE 2

EXPLANATION OF FIGURES

7 Estrus. Incubated one hour. × 250.

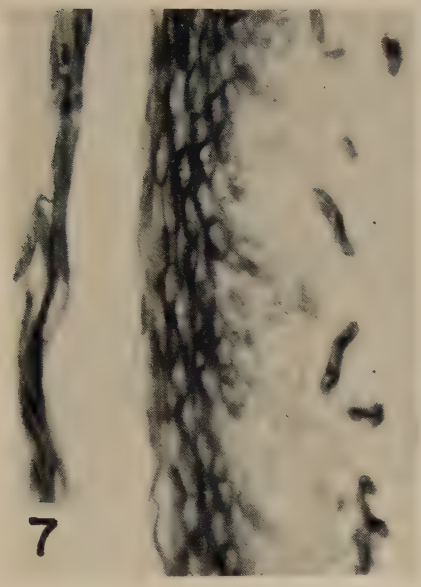
8 Estrus, showing deep discontinuous zones of cobalt sulfide deposition. Incubated one hour. × 250.

9 Metestrus. Incubated one hour. × 200.

10 Spayed 5 weeks. Incubated one hour. × 150.

11 Same animal as figure 10, adjacent region showing mucified surface cells. Incubated one hour. × 150.

12 Spayed animal injected with 0.5 mg estradiol benzoate. Incubated one-half hour. × 250.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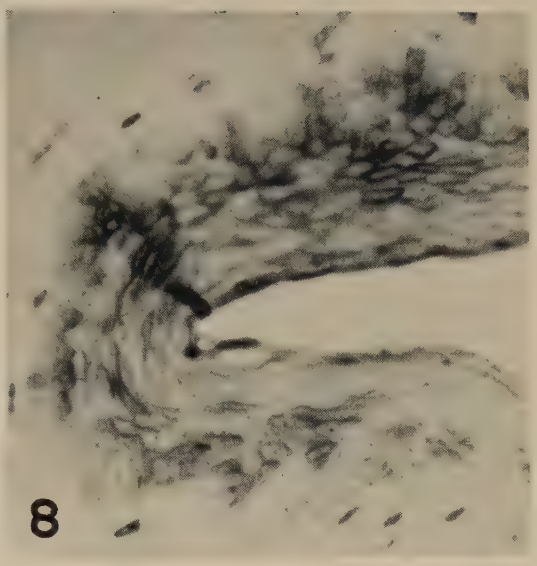
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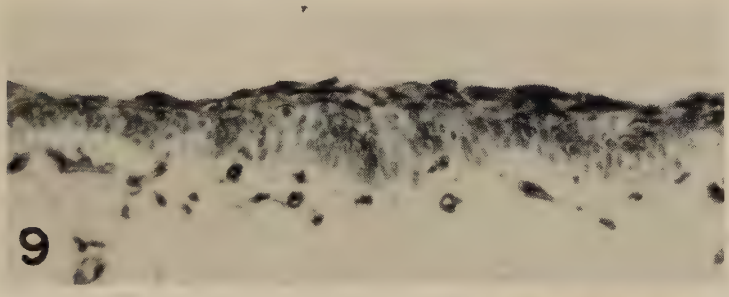
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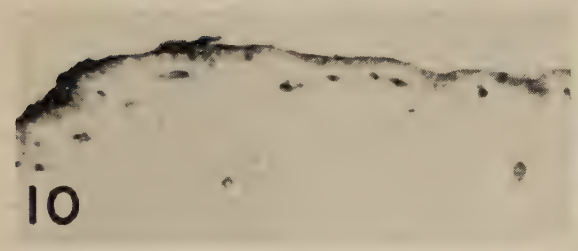
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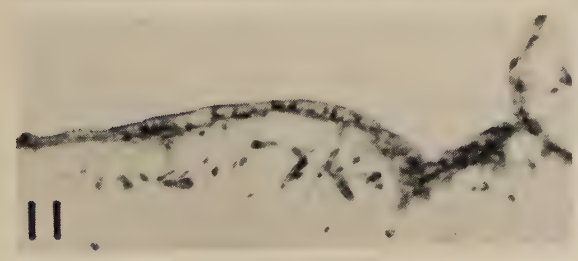
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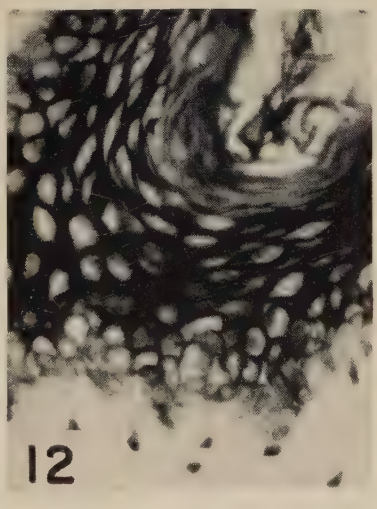
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EFFECT OF INJECTIONS OF SOLUBLE ANTIGEN ON THE SPLEEN SIZE AND ANTIBODY PRODUCTION IN CHICKENS[1]

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TWO FIGURES

Young mammals of numerous species have been found to be less effective producers of antibodies than adults of the same species. This problem of the relative difference in the ability of young and adults to produce antibodies has been studied most extensively in man, rabbits and guinea pigs (reviewed by Baumgartner, '34). Wolfe and Dilks ('47) found this to be equally true of chickens. Chicks less than 5 weeks old often failed to produce detectable precipitins.

Very little work has been done on the changes in the spleen of the young animal after the injection of soluble antigen, although a great deal of interest has centered around the changes in the spleens of adult mammals after such injections. Rich, et al. ('39) pointed out that infants are deficient in their ability to produce antibodies and that acute splenic tumor rarely becomes so well developed during infections in infants as in older children or adults. Gross ('19) in a study of the human spleen in relation to disease states that the spleens in middle age show reactive phenomena to disease in contrast to the behavior of young spleens. The spleen's

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greatest response to an infection seems to occur in middle life in man.

Changes in the macroscopic and microscopic appearance of the spleen after antigen injection have been reported many times. Dominici ('00) reported that rabbits injected with a culture of "Eberth's bacillus" (*Eberthella typhi*) showed numerous mitoses in the splenic follicles from 7 to 24 hours after the injections and spleen volume increased up to 5 times. Hellman and White ('30) stated that increase in volume of a rabbit spleen occurs only after injection of a particulate antigen, but Rich ('35) found that non-particulate antigens such as egg white and mammalian sera caused an increase in splenic size. He noted marked mitotic proliferation and enlargement of the mononuclear cells at the margin of the Malpighian bodies. Conway ('38, '39) studied the effect of injection and reinjection of rabbits and guinea pigs with *Bacterium monocytogenes* and emphasized the very rapid alterations in the lymphoidal tissue, in some cases within a matter of hours. Ehrich and Voigt ('34) studied changes in the lung, liver and spleen of rabbits after injection and reinjection of *Staphylococci.* The spleen increased in weight after both injections. The liver increased slightly and the lung greatly after the second injection. The authors considered the lung change to be mesenchymal.

The present paper correlates the changes observed in the spleen after injection of antigen with the age of the chicken and with antibody production.

MATERIALS AND METHODS

The chickens used in these experiments were of both sexes and varied in age from 1 day to 12 weeks. Some adults of about one year were used. The majority were hybrids between White Leghorns and either Barred Rocks or New Hampshires, although a few were pure-breds of one of the three breeds.

Most of the birds were injected with either crystalline beef albumin or undiluted beef serum. A few adults were

injected with either horse or rabbit serum. All of these antigens have yielded good antibody response in our laboratory. For simplicity in carrying out the experiments, the amount of antigen injected was the same for birds of the same age group. No correction of the amount injected was made for variation in weight within an age group. Chickens under one week of age were injected subcutaneously or intraperitoneally; all others were given intravenous injections. Preliminary data in our laboratory have indicated that the route of injection has little, if any, effect on the results in the type of experiments that we were performing.

The chickens were bled by cardiac puncture (after 12 to 18 hours of starvation) at varying intervals starting two days after the last injection and continuing to 30 days. The spleens were removed immediately after bleeding the chickens and after being cleaned of excess fat and mesentery were weighed in a closed balance. Twelve or more days after bleeding the presence of antibody was determined by the interfacial (ring test) method. (Doubling dilutions of antigen are made in small serological tubes with 1.8% saline solution, and 0.1 ml of antiserum is carefully layered beneath 0.5 ml of each antigen dilution; the titer [endpoint] of these antisera is defined as the highest dilution of antigen at which a distinct ring is visible at the interface.)

Since it is not possible to compare the spleen weight before and after antigen injection in the same bird it was necessary to use controls, and in view of the great variability in spleen size a large number of individuals were needed to determine average values with any precision. Fortunately we had available as control data a large number of spleen measurements on normal (uninjected) chickens of all ages (Norton and Wolfe, '49). In addition a small number of chickens was injected with saline and their spleens showed no enlargement.

In the control data for the adult birds the average spleen weight was 0.094% of the body weight in males and 0.105% in females. The increase or decrease in spleen weight of the injected chickens was recorded as a percentage increase

or decrease from the average control value for a bird of that sex and weight.

In young chicks from 60 to 1200 gm a different procedure was used. The spleen weight is not the same proportion of body weight as the latter increases in size. However, we found, in accordance with the usual results of studies on heterogonic growth, that a linear relationship existed between the logarithms of the spleen weight and the body weight. The equations used were of the form $Y = y + b(x - \bar{x})$, where $x =$ log body weight, $Y =$ log spleen weight, $\bar{x}$ and $\bar{y}$ are the mean values of the log body and spleen weights respectively and b is the regression coefficient determined by the usual least squares procedure. The values, based on data from over 700 birds, were as follows:

	MALES	FEMALES
no. of birds	337	418
$\bar{x}$	2.436	2.410
$\bar{y}$	2.550	2.581
b	1.317	1.305

The difference in the regression coefficient is not statistically significant. The spleen weight is slightly greater for a given body weight in females than in males, as was the case in the adults, but the difference is not statistically significant.

The spleen weight for each injected chicken was expressed as a percentage increase or decrease in spleen weight from the expected weight as given by the allometric equation for a bird of that weight.

RESULTS

Spleen weight and amount of antigen injected

The results of 4 experiments on 6- and 7-week-old chicks given varying amounts of antigen are shown graphically in figure 1. In experiment XIII 20 chicks of about the same

weight (530 gm) were injected with 1 ml of beef serum which is equivalent to the average of 1.9 ml per kilogram body weight. As can be seen from the graph only a slight increase was found in spleen size from 6 to 42 hours after injection.

In experiment VI (45 chickens) and experiment XIV (39 chickens) larger amounts were injected, about 2.6 and 3.9 ml per kilogram body weight respectively. The chickens responded remarkably to this dose and the spleens can be seen

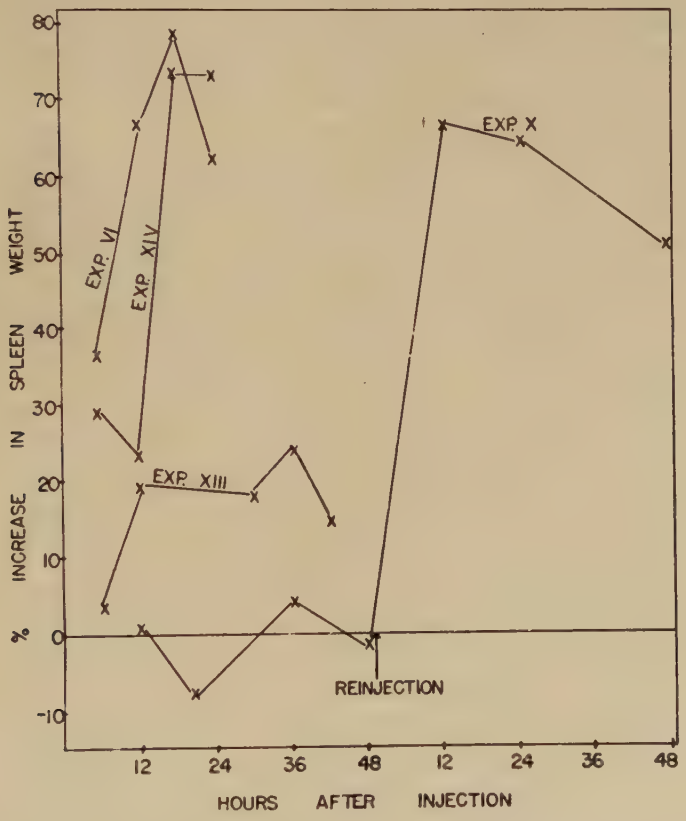

Fig. 1 Per cent increase in spleen weight after injection of different quantities of beef serum antigen.

Experiment VI. Six weeks old. 2.6 ml/kg.

Experiment XIV. Six weeks old. 3.9 ml/kg.

Experiment XIII. Seven weeks old. 1.9 ml/kg.

Experiment X. Six weeks old. 0.49 ml/kg, reinjected 0.98 ml/kg 48 hours later.

to have reached a size about 75% greater than normal in 18 hours.

In much experimental work on antibody production in animals more than one injection is commonly given. In view of this it was of interest to determine the increase of the spleen after reinjection. In experiment X (fig. 1) each of 28 chickens was given an initial injection of 0.49 ml beef serum per kilogram body weight. As was to be expected no change in spleen size was found up to 48 hours at which time a second injection, double the initial amount, was given. The response to this injection was very rapid, and within 12 hours the spleen had reached its maximum size, an average of about 75% above the controls.

Two injections are apparently much more effective than the same amount given as a single injection. Although the first injection did not itself cause any noticeable increase in the spleen size it made the bird much more responsive to the second dose. This is in accord with the common belief that the same amount of antigen is more effective when given in a series of injections than when given all at once.

Increase in spleen weight in chickens of various ages

One-day-old chickens did not have any significant increase in spleen weight. The data for one-week olds are insufficient, but indicate that the spleens have increased in size on the 5th day after injection. Experiment XIX on two-week olds is complicated by the unusually large spleen of the chickens used as controls for this experiment. The spleen size of the controls in this experiment was found to average about 50% more than the expected spleen size for chickens of that body size from previous control data. Further, no peak of increase in spleen size can be seen in the injected animals. It seems, therefore, that the spleens of these chickens did not respond greatly to the injection of antigen. It is possible that the large spleen size of the controls indicates that some infection might have been present in these chickens and that the spleens had enlarged in an immune reaction.

Beginning with the experiments on three-week-old chickens, all of the age groups showed a sudden, rapid enlargement of the spleen followed by a slower return to normal. Figure 2 shows this graphically for 3-, 4-, 6- and 12-week-old chicks. The maximum of spleen size is reached in all age groups by the 5th day. The spleens of all of the age groups have returned to within the normal range of variation before the

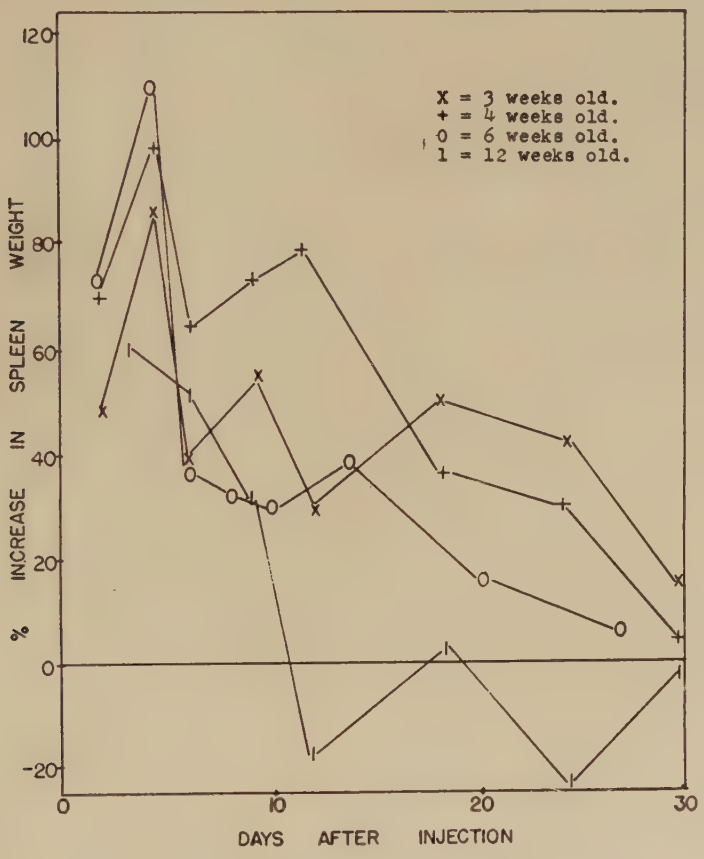

Fig. 2 Per cent increase in spleen weight after injection.
X, beef albumin 40 mg/kg.
+, beef albumin 40 mg/kg.
0, beef serum 2.8 ml/kg.
1, beef albumin 50 mg/kg.

30th day after the last injection of antigen. The maximum response is seen to be quite similar in the various ages except perhaps for being less in the 12-week olds. The same percentage increase, of course, represents a much greater actual increase of tissue in the older chicks. The apparently more rapid return to normal in the 12-week-old chicks may be nothing more than a consequence of a smaller percentage increase at the maximum.

TABLE 1

The effect of injection of various antigens on the spleen weight of adult chickens

NUMBER OF CHICKENS	SERIES OF INJECTIONS	DAYS AFTER LAST INJECTION	% INCREASE IN SPLEEN WT.	ANTIGEN INJECTED
19	1[1]	6	54.6	Beef, deer or rabbit serum
8	1	7	48.4	Rabbit serum or human globulin
8	1	8	18.1	Human globulin or albumin
9	1	9	27.8	Beef albumin
2	2[2]	5	98.3	Beef albumin
2	2	6	33.5	Beef serum
11	2	7	106.5	Beef serum
1	2	8	101.3	Insect serum

[1] Two or three injections on alternate days, totalling between 1.50 and 3.0 ml of undiluted crude serum or 4% of purified antigen.

[2] Two or three injections on alternate days administered approximately one month after first series. Quantity injected similar to first series.

A striking difference in the young animal as compared with the older is the variability of response of the former. The standard deviation of the per cent increase in the spleens of 3- and 4-week-old chicks was 75.6 while for 5-, 6-, and 7-week-old chicks it was 46.6. (This agrees with the precipitin response of these age groups reported by Wolfe and Dilks, '47.)

Table 1 shows data on the average increase in spleen weight in adult chickens. These birds were injected with several different kinds of antigen; their spleens were significantly larger than those of uninjected animals.

Water content and macroscopic appearance of spleens from injected chickens

There is a possibility that the increase in spleen weight of injected animals is due to oedema. To check this dry weights were compared. Six 14-week-old males were injected with 40 mg of beef albumin/kg and reinjected 48 hours later with 60 mg/kg. Six additional uninjected chickens were used as controls. The spleens were removed three days after the last injection and the wet weight determined. The spleens were then sliced and the pieces dried for 24 hours at 75 to 85°C. The wet and dry weights and the dry weight as a per cent of the wet weight were analyzed. There is no appreciable difference in the dry weight percentage of the wet weight between experimentals and controls, thus showing that the increase in spleen weight is not due to an increase in water content without a corresponding increase in the amount of tissue in the spleen. The data also lead to the conclusion that the increase in spleen weight in injected animals is not primarily due to an increase in red cells whose low water content would have increased the per cent dry weight of the spleens from injected animals.

Injected spleens were found upon macroscopic examination to have a typical pattern of appearance distinguishing them from normal spleens. The capsule had a swollen, slightly mottled appearance at the peak of the increase in spleen size. Numerous secondary follicles were prominent on the cut surface of the spleen. The margins of the white pulp were indistinct and had spread out into the red pulp. Upon microscopic examination increased numbers of lymphocytes were found to be responsible for much of the hyperplasia. The problem of comparing normal chicken white cells

with those of mammals, a problem not yet completely solved (Olson, '37), complicates any histological study of the effect of injection of antigen on the spleen.

Correlation of the spleen increase with precipitin titer

The comparison of the change in spleen size with antibody titer is of great importance in the development of the theory that the spleen is associated with antibody production in the chicken. Bjørneboe and Gormsen ('43) found a correlation of spleen size with the per cent of antibody protein in the serum of immunized rabbits.

In the present experiments, recorded in table 2, the per cent increase in the size of the spleen was correlated with the interfacial titer of the serum at that time. Instead of using the greatest dilution of antigen in which a reaction was observed as the titer of an individual serum, the successive dilutions of antigen were numbered consecutively. The number of the last tube in which a reaction was observed was used as the titer of that serum in all statistical considerations of the data. This method is equivalent to using the logarithm of the antigen dilution. When beef serum was used as the antigen the first tube contained antigen diluted 1:100. In the case of beef albumin the first tube contained 64 gammas of antigen N. The N content of these two tubes is approximately the same.

In correlating the increase in spleen size with titer only those titers were used which were obtained while the spleens were increasing in size or were at the peak of their increase. Since the antibody titer is still found in the serum after the spleens have returned to normal size, no correlation could be expected to hold after the spleens have started to decrease in size.

The calculated coefficient of correlation for the per cent increase in spleen size and titer was 0.25. This correlation, though positive, is small. However in view of the variability of both spleen size and antibody titer a high correlation would not be expected.

TABLE 2

The spleen weights and interfacial titers of injected chicken

EXP. NO.	AGE	MG/KG OR ML/KG [1]	TYPE [2] OF EXP.	DAYS AFTER INJECTION														
				1	2	3	4	5	6	7	8	9	10	12	14	18	24	30
	days																	
XVI	1	5.8 ml	W (52)[3]		16													
XVII		5.4 ml	W (43)						21									
	weeks																	
XI	1	3.5 ml	W (15)					74										
XVIII	2	8.0 ml	W (43)						51									
XIX		120.0 mg	W (44)		23		38		49			21		57		34		
			T (35)		0		0		4			4		0				
XX	3	140.0 mg	W (92)	60	54	96	33	63	52	36	30	28						
			T (72)			3	0	5	10	11	9	10						
III		120.0 mg	W (56)		50		84		38			56		28		48	40	16
			T (42)		0		5		9			6		1.5		0		
VIII	4	7.5 ml	W (61)	59	82	47		47		21			31					
			T (61)	0	0	0		4		8			2					
II		120.0 mg	W (58)		69		99		62			67		75		32	26	3
			T (50)		0		5		12			9		4		0	0	
XXI	5	2.7 ml	W (24)		58		53		70		60		30	33	26	20		
			W (31)				6		12		10		9	8	7	3		
XII	6	2.7 ml	W (90)		71		109		36		32		28		37	15	6	
			T (90)		1		6		10		9		6		5	2	0.5	
IV		50.0 mg	W (28)			113			41			33		21		17	10	14
VII	7	1.4 ml	W (24)	31	51	58				26								
			T (18)	0		9				12								
XXII		1.5 ml	W (16)			73		57				41		20				
			T (16)			7		12				12		5				
I		3.0 ml	W (28)	91	102	83	111	98	73	56								
			T (20)			0	2	11	12	13								
V	12	50.0 mg	W (21)			68			48			29		—18		4	—21	—1

[1] Milligrams of beef albumin or milliliters of undilute beef serum injected per kilogram body weight.
[2] W = spleen weight in milligrams; T = average interfacial titer expressed in tube number.
[3] Number in parentheses is total number of animals used: these were approximately equally divided among the various experiments.

Titrations were made on sera from chickens, two weeks of age and older. The lowest titers were obtained in experiment XIX on the two-week chicks and these birds did not show any noticeable increase in spleen size. The next lowest titers were in experiments VIII and III on 4- and 3-week-old chickens. However, the titers obtained in two other experiments on chickens of the same ages (II and XX) show that the same maximum titer can be produced in these two age groups as in older animals. This agrees with the findings on the maximum per cent increase in the size of the spleens of these groups. In general a more rapid development of antibody titer is noticed in the older age groups. This corresponds to an apparently more rapid increase in the size of the spleen of the older animals. Again, this change takes place at about 5 weeks of age.

Comparing the peak of the titer in all of the experiments with the peak of the spleen size it is found that the peak of spleen size occurs about two days before the peak of antibody titer is reached. This may be one reason that the correlation observed between spleen size and titer was not greater.

DISCUSSION AND CONCLUSIONS

It has long been known that chickens are good precipitin producers and that they share this type of response to the injection of foreign protein with some of the mammals. The production of antibodies, of which precipitins are a readily demonstrable type, is one of the most important mechanisms of the body in protection against disease. The present paper shows a further similarity of the response of chickens and mammals in antibody production — the spleen enlarges similarly and this enlargement can be correlated with the rise in antibody titer.

The spleens of chickens younger than three weeks respond irregularly to the injection of antigen, showing no regular increase in size. This is correlated with the lower antibody titers observed. Although the same average maximum titer as in older chickens may be reached in 3- and 4-week-old

animals, a measurable titer appears more slowly and disappears more rapidly than in older chickens. The spleens of 3- and 4-week-old chickens are also more variable than older ones in their response. These facts bear out the results of Wolfe and Dilks ('47) on the age at which precipitins were found in the sera of all of the animals.

There is some, but not striking, correlation of individual increase in spleen size with interfacial titer but the variability of the normal chicken spleen is so great that very high correlation would be unlikely. One difficulty here is that the response of the spleen is so rapid that it may have passed its maximum size before the maximum titer is found in the blood. Higher correlation might be obtained in hyperimmunized chickens (three or more series of injections) where the results on adult spleens have indicated the increase in spleen size is maintained for a longer period. It is a common practice for many serologists to use hyperimmunized animals for study. The differences in results that may arise from using animals with different periods of immunization should be emphasized. An additional problem in correlating spleen size with titer in chickens of varying ages is the difference in the period of response of the spleen in these groups. The spleen responds more slowly in younger chickens. The significance of this is not understood although it may be related to a general immaturity of the antibody producing mechanism of young animals.

The increase in the size of the spleen is due to an increase in the number of cells but no definite information as to relative increase of different types of cells can be given at this time. The increase in size is related to a proliferation of the white pulp and an increase in the number of secondary nodules.

SUMMARY

1. The minimum amount of antigen required for the response of the spleen in young chickens is about 150 mg of protein/kg body weight or about one-third this amount if given in two injections.

2. The response of the spleens of chickens younger than three weeks is questionable.

3. There was considerably more variation in the degree of response of the spleens of chickens 3- and 4-weeks-old than in the older groups.

4. Increase in spleen weight in the injected animals is due to real tissue increase not just a higher water content, as shown by comparison of dry weights of experimentals and controls.

5. The capsule of the injected spleens showed numerous secondary follicles, and the capsule had a swollen and slightly mottled appearance.

6. No increase in the size of the liver corresponding to the increase in the spleen was found.

7. A correlation of individual increase in spleen size and antibody titer was found. In all groups the peak of the titer was reached about two days after the peak of spleen size.

The authors wish to thank Professors W. W. Cravens and W. H. McGibbon of the Poultry Husbandry Department and Dr. C. A. Herrick of the Zoology and Veterinary Science Departments for supplying many of the chickens used in this study.

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A METHOD FOR ROUTINE DETECTION OF RADIO-PHOSPHATES AND OTHER RADIOACTIVE COMPOUNDS IN TISSUES. THE INVERTED AUTOGRAPH

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SIX FIGURES

INTRODUCTION

The classical and still widely used method for detection of radio-elements consists of placing sections of a radioactive tissue in contact with a photographic plate. This technique is crude and usually does not permit the assignment of the reactions of the photographic plate to definite histological structures. A step forward was taken when section and emulsion were kept in close contact throughout exposure, development and mounting, in such a way that the structures and the dark reactions due to their radioactivity could be visualized at the same time under the microscope. This was first done by coating histological sections with photographic emulsion and then staining through the emulsion (Bélanger and Leblond, '46). Later, the technique was made more convenient by staining the sections before coating them with emulsion (Leblond, Percival and Gross, '48). However, with bones and teeth, pre-staining with the usual acid dyes resulted in a loss of phosphates from the sections; and, therefore, the detection of P^{32} could not be carried out with accuracy. A new method has been developed by which the section may be stained after exposure and development; and, therefore, the loss of phosphate in staining would occur after the autograph had been obtained and would no longer be a disadvantage.

METHODS

Preparation of histological sections

The mineral salts of bones and teeth are retained when the tissues are fixed in 10% neutral formaldehyde, absolute alcohol or the following mixture, by which a better fixation is obtained:

95% Alcohol	75 cm³
Formaldehyde, 40%, neutral	25 cm³

Paraffin embedding after dehydration in dioxane as usual was used when the soft bones of newborn animals were studied. After birth, the bones of the young animals were cut in celloidin, according to a modification of Bloom's method as described by Leblond, Wilkinson, Bélanger and Robichon ('50). The duration of embedding was shortened by the following procedure. The tissues coming from the bath of alcohol-ether were placed overnight in 10% low-viscosity nitrocellulose embedding solution (no. M 4700 of Randolph Products Co., Carlstadt, N. J.). The next morning, the tissues were centrifuged in this solution for one hour at 1700 r.p.m. They were then placed successively in solutions containing 20% and 30% of nitrocellulose and centrifuged for one hour in each. Finally the tissues were placed in fresh 30% solution in shallow dishes and left to evaporate slowly under a bell jar until they became sufficiently hard for unmolding and trimming. This required between 2 to 7 days, depending of course on the size of the pieces.

The sections (ca. 10 μ) were attached to chemically clean glass slides coated with a thin layer of Mayer's albumin-glycerine by smearing with the help of another slide. The supporting medium was dissolved out with alcohol-ether and the slides were dipped twice in a 1% solution of celloidin and left to dry overnight.

Matrix films (Eastman Kodak Co.) were used as a source of emulsion. A 4×5 inch sheet supplies enough emulsion to coat 6 microscope slides. Under a yellow-green safelight (series OA) at three feet, each film sheet was cut in two halves

which were placed for about a minute in a 600 cm^3 beaker filled with distilled water at room temperature. The hydrated emulsion was then scraped off the base with a clean glass slide and transferred to a 50 cm^3 beaker placed in a water bath at 38°C. By keeping the beaker in the bath for 10 minutes, an even melting of the emulsion was obtained. The slides were then coated with the emulsion, using a brush previously made by folding over a clean microscope slide, several layers of bandage gauze maintained in place with cellulose adhesive tape. The brush was soaked in the emulsion

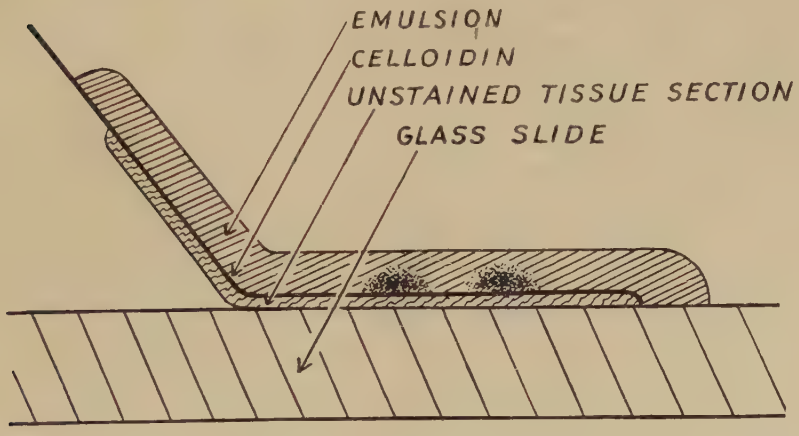

Fig. 1 Disposition of the components of the preparation, previous to inversion.

and carried over the surface of each slide *once* and with a uniform slow motion, a minimum of pressure being applied. After coating each slide, the brush was replaced in the melted emulsion kept in the water bath. The coated slides were laid on a leveled glass plate where they were dried in approximately 15 minutes, with the help of a gentle air stream generated by a fan. At this stage, the various components of the preparation are arranged as shown in figure 1. The dry slides were placed in individual containers (slide mailers, Clay Adams Co., Inc.) which were stacked in large boxes containing a dish of calcium chloride. These boxes were

wrapped twice with black paper, sealed with cellulose adhesive tape and placed in a refrigerator at 4°C. for the duration of exposure of the emulsion to the tissue radiations. The dry slides can also be placed in black plastic boxes, the edges of which are sealed with electrician's tape.

It was found imperative that, during all subsequent operations in aqueous media, the emulsion be prevented from swelling and wrinkling. This was best accomplished by maintaining the temperature of the solutions at 10°C. or below.

For developing and fixing, horizontal staining dishes of an approximate capacity of 225 cm^3 and containing respectively the developer, rinse and fixer were immersed in a bath of melting ice. The slides were carried through these dishes with the help of glass trays, the wire holders of which had been given a triple plating of silver.

The slides were developed for 10 minutes at 4–5°C. in Kodak Dektol diluted with an equal volume of distilled water. No apparent improvement in resolution was obtained with the so-called fine grain developers. Fixation was carried out for 10 minutes in Kodak acid fixer with hardener. The presence of hardener in the fixing bath is important since, when it was omitted, the fragile emulsion-tissue combination tended to twist and roll when removed from its glass support.

After washing the sections for 30 minutes the operation of *inversion* was carried out under water in a flat white bottomed dish at 8–10°C. With the help of a razor blade placed in a holder, the emulsion-tissue combination was lifted from the glass slide and seized with a pair of forceps to be turned emulsion face down and thus applied on a clean glass slide slightly coated with albumen-glycerin adhesive. The emulsion-tissue combination was smoothed down on its new support with a wet sable brush, care being taken not to touch the histological sections.

These slides were then placed in a glass tray in front of an electric fan which dried them rapidly.

After 3 to 6 hours, the edges of the emulsion-tissue combination were stuck to the glass with 1% celloidin applied

with a sable brush kept in alcohol-ether between operations. This step prevents penetration of the dyes under the emulsion during staining. It is not necessary with celloidin sections.

After drying the slides overnight, *staining* was done with safranin or basic fuchsin. The lattter dye provided a satisfactory monochromatic histological background for microscopic study or photography of the preparations.

Paraffin section autographs were taken to distilled water for approximately one minute and transferred to 0.1% aqueous fuchsin for 4 minutes. Differentiation and dehydration

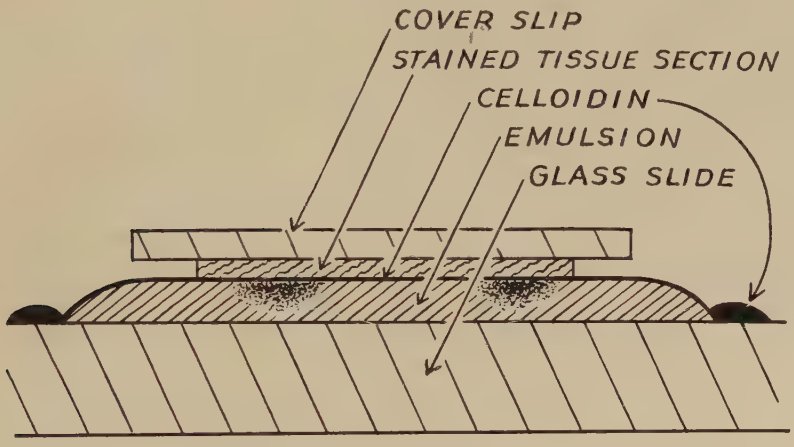

Fig. 2 Disposition of the components in the finished inverted preparation.

were achieved through distilled water, 95% and absolute alcohol, and toluol, before mounting in balsam (fig. 2).

Celloidin section autographs were also stained with basic fuchsin but the dry celloidin had to be made permeable. As soon as the inverted sections were dried they were carried through successive baths of 50%, 80% and 95% alcohol for a period of two minutes in each. They were then placed in oil of origanum [1] for 5 minutes, following which they were taken to 95% alcohol for 4 minutes and then through two

[1] The most satisfactory for the present purpose is made by Eimer and Amend, New York.

minute baths of 80% and 50% alcohol, and finally placed in distilled water for 5 minutes. They could then be stained either rapidly in a 0.1% aqueous solution of basic fuchsin for 8 to 10 minutes, or slowly, but better in 0.02% solution of the same dye for about 90 minutes. The sections were then passed rapidly through two changes of absolute alcohol, followed by two minutes in a third absolute and two minutes in each of three toluenes, following which the sections were mounted with cover slips and Permount as described previously (fig. 2).

DISCUSSION

Several techniques have recently been proposed to obtain preparations with an intimate contact between emulsion and specimen, which H. Yagoda calls elegantly "integral" radioautographs:

1. The "coated autograph" techniques (Bélanger and Leblond, '46; Leblond, Percival and Gross, '48), by which the fluid emulsion is spread over the sections. In a variant of this method, a stripping emulsion is placed over the section. It is the "stripping film technique" (Pelc, '47; MacDonald, Cobb and Salomon, '48; Boyd and Williams, '48).

2. The "mounted autograph" technique (T. C. Evans, '47; Endicott and Yagoda, '47) in which the histological sections are stuck directly on the emulsion surface of a photographic plate.

In our opinion, the inverted autograph technique possesses several advantages over the previously described methods.

For general use, it permits mass production with little loss of material, thus making practical the use of radioautography with serial sections. The possibility of working under the relatively bright illumination from the series OA yellow-green filter adds to the ease and efficiency of operations. The present technique has a definite advantage over the initial coated autograph technique in permitting routine and easy post-autographic staining. For the study of the mineral portion

of bones and teeth (figs. 3, 4, 5, 6), staining before coating with emulsion is a hazard which often results in loss of at least part of the tracer element.

It is obvious also that the stained sections which have to go through the developer and fixer will lose some of their color, a defect which makes for uneven results and which is obviated by the present method. Basic fuchsin is now used routinely with bone and tooth preparations (figs. 3, 5, 6), but a weak haematoxylin will take well if it seems desirable.

The mounted autograph technique, which also obviated the loss of phosphate has two disadvantages: (1) the developer has to reach the emulsion after it has passed through the histological section, a condition responsible for uneven and spotty development, especially in the case of celloidin infiltrated sections which when dry are impenetrable to aqueous solutions; (2) the emulsion takes up much stain.

The inverted autograph technique as presently described does not, on the other hand, fulfill all requirements for radioautography at the cytological level. The resolution below 100 diameters is good only when the radiating areas are widely segregated such as in the case of the cartilage cells in figure 5.

For studies of bones and teeth which could be sectioned with a microtome (figs. 3, 4, 5, 6), the inverted autograph technique has been found fully adequate and reliable.

SUMMARY

A modification of the coated autograph technique for the localization of radioactive elements in tissues is described under the name of "inverted autograph."

Kodak Matrix emulsion is painted over the unstained celloidin-coated histological section. After exposure, development and fixation, the combined emulsion-celloidin-tissue preparation is inverted and the section is stained. The advantages are: (1) possibility of mass production; (2) working in semi-brightness; (3) retention in the sections of P^{32} present as mineral salt; (4) easier and better staining.

ACKNOWLÈDGMENTS

This technique was devised during the course of investigations on the growth of bones and teeth, under the leadership of Dr. C. P. Leblond of McGill University.

Technical advice was provided by G. Graham, Director of Technical Operations at the National Film Board of Canada and H. W. Holmes, Head of the Biological Photographic Laboratory of the Department of National Health and Welfare.

The skilled help of Mrs. Patricia Brown, Miss Jean MacKinley and Mrs. Cécile Bélanger in the preparation of the histological, autographic and photographic material is acknowledged.

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PLATE

PLATE 1

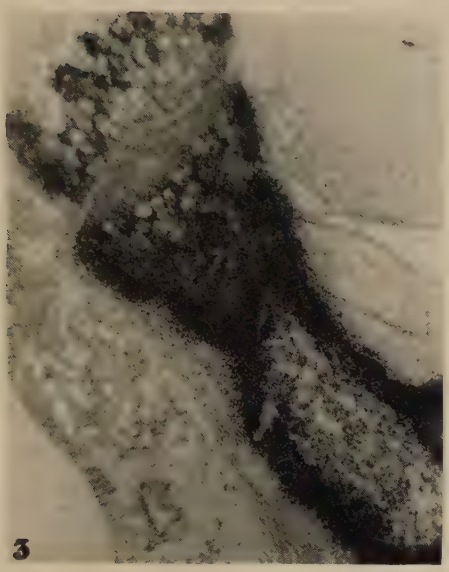
EXPLANATION OF FIGURES

3 Portion of thoracic vertebra in a newborn hamster, sacrificed one hour after a subcutaneous injection of radiophosphorus. The radio-element is present at the border of the distal enlarged cartilage cells and all over the diaphyseal bone except at the point of entry of the nutrient artery. × 100, paraffin section, basic fuchsin stain.

4 Portion of the ossifying proximal tibial epiphysis in a 50 gm rat, sacrificed one hour after a subcutaneous injection of radiophosphorus. The radio-element has given an autographic image at the periphery of the distal enlarged cartilage cells as in figure 3 and all over the bone spicules along with osteoblasts may be seen. × 100, celloidin section, azure II — eosin stain.

5 Portion of the head of the femur in a frog tadpole, sacrificed one hour after a subcutaneous injection of radiophosphorus. The newly formed cartilage cells in the upper left corner do not fix radiophosphate, while the older ones show deposition at first light and peripheral and lower down, more abundant and distributed over the whole area of the cell. This reaction over the cells has never been seen with mammalian cartilage. × 400, paraffin section, basic fuchsin stain.

6 Section of a developing cusp in the first molar of a 4-day hamster, sacrificed one hour after a subcutaneous injection of radiophosphorus. The radio-element is present in both enamel and dentine, a larger concentration being apparent in the latter layer. × 100, celloidin section, basic fuchsin stain.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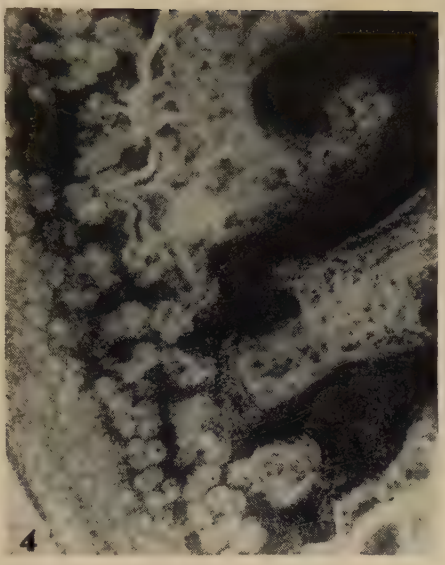
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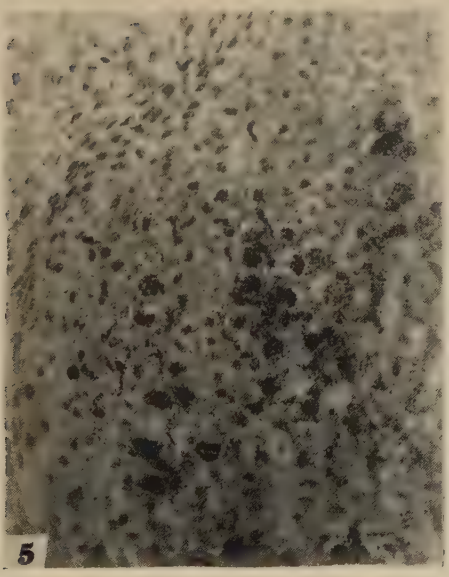
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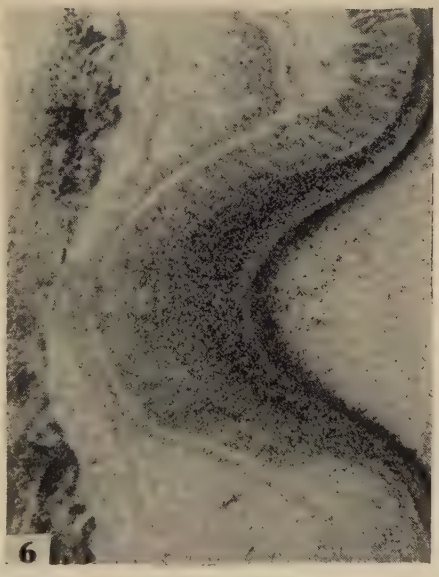
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THE SUBMAXILLARY GLANDS OF SHREWS

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FIVE FIGURES

Despite the fact that folklore on several continents attributes a poisonous quality to the bite of shrews, it was a surprise to find that an extremely toxic substance could actually be obtained from the salivary glands of at least one species of shrew, *Blarina brevicauda*. It has been shown elsewhere (Pearson, '42) that from the submaxillary glands of a single specimen of this mouse-sized mammal enough poison can be extracted to kill by intravenous injection nearly 200 white mice or several cats and rabbits. One of the clues leading to the discovery of this substance was the presence in the submaxillary glands of *Blarina* of an unusual segment of granule-filled cells in the tubules between the intralobular ducts and the terminal acini. I have recently endeavored to determine which species of shrews produce a poison and whether they alone possess the curious segment mentioned above.

THE SUBMAXILLARY GLANDS OF BLARINA

After appropriate histological preparation one finds in the submaxillary tubules of *Blarina*, the common short-tailed shrew of the eastern United States, a segment of cells that are greatly distended by large acidophilic granules. These segments radiate conspicuously from the cluster of secretory ducts in the center of each lobule of the gland. The granule-filled cells are immediately adjacent to the striated cells of the secretory duct and form a duct which appears to connect directly with the terminal acini without the interposition of

an intercalated duct. The duct of granule-filled cells may, therefore, represent an hypertrophied intercalated duct, or it may represent an enlargement and specialization of part of the secretory duct or terminal acinus accompanied by a reduction of the intercalated ducts (figs. 1–5).

In preparing sections of the salivary glands of *Blarina* the following fixatives were used: Bouin's fluid, Zenker-formol, 10% formalin, and formol-sublimate solution. Staining was with Delafield's hematoxylin and eosin, Mallory's triple stain, or Heidenhain's azan modification of Mallory's stain. Dehydration was through alcohol and xylol, or dioxane. The enlarged segment of granule-filled cells was revealed by

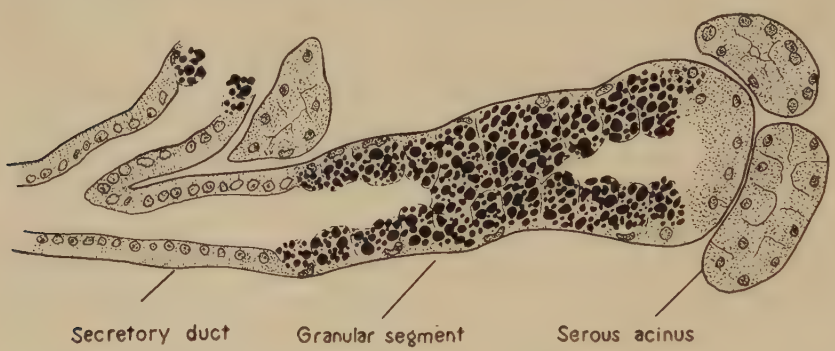

Fig. 1 Diagram of submaxillary tubule of *Blarina brevicauda*. × 410.

all combinations of these methods. The most striking preparations were fixed in Zenker-formol and stained with Mallory's triple stain. This treatment reveals the content of the cells of the granular segment as large, distinct, orange-red granules, some of them larger than nuclei (fig. 3). The granules stain red with azan and pink with eosin, but are not always clearly granular with the latter stain. Bouin's fixation followed by Mallory's stain produce variable results. Occasionally the granules stained orange-red but usually appeared as pale blue granules or a pale vacuolated mass. In two of the 22 *Blarina* studied the granular segment was not found. These were the only estrous females sectioned. This fact

may be of importance in the light of recent findings concerning the effects of hormones on the submaxillary glands of rodents (Raynaud and Rebeyrotte, '49; Junqueira et al., '49). These workers have found morphological and enzymatic differences between the sexes, differences that can be affected by castration and by injections of sex hormones.

THE SUBMAXILLARY GLANDS OF OTHER SHREWS

The submaxillary gland of the European water shrew, *Neomys fodiens,* has been described and illustrated by Schaffer ('08). He found a granular segment clearly similar to that described above, but in which, judging from his illustrations, the granule-filled cells are not as enlarged and the secretory granules somewhat smaller than in *Blarina.* After preservation in formalin the granules in his sections stained brightly with eosin, chromatrop 2R, orange G, and darkly with Heidenhain's iron hematoxylin. They did not stain with Delafield's hematoxylin or mucicarmine.

Schaffer ('08) also sectioned submaxillary glands of two other European shrews, *Sorex araneus* and *S. alpinus,* and reported that no granular segment was present.

I have cut sections of the submaxillary glands of one or more individuals of the following kinds of shrews and find that they contain a granular segment similar to that found in *Blarina* and *Neomys: Sorex fumeus fumeus, S. pacificus pacificus, S. p. sonomae, S. palustris navigator, S. sinuosus, S. trowbridgii montereyensis, S. vagrans vagrans, S. v. amoenus, S. v. halicoetes,* and *Cryptotis parva.* The staining reaction in the tubules of these species is similar to that in *Blarina,* but the tubules differ slightly in that the granular segments are not as enlarged as in *Blarina,* and the nuclei of the granule-filled cells in *Sorex* are fairly large and spherical and are not usually flattened or located close to the bottom of the cell as in *Blarina.* In these respects the above members of the genera *Sorex* and *Cryptotis* resemble *Neomys.*

Sections were also cut of the submaxillary glands of three non-breeding specimens of *Sorex cinereus cinereus* and a sin-

gle female of unknown reproductive condition of *Sorex bendirii bendirii*. No granular segment was found in these forms. Also lacking the granular segment were the common mole (*Scalopus aquaticus aquaticus*), the shrew-mole (*Neurotrichus gibbsii gibbsii*), marten (*Martes caurina*), white-footed mouse (*Peromyscus leucopus noveboracensis*), meadow mouse (*Microtus pennsylvanicus pennsylvanicus*), pine mouse (*Pitimys pinetorum scalopsoides*), muskrat (*Ondatra zibetheca macrodon*), and albino house mouse (*Mus musculus*).

SALIVARY EXTRACTS

Injections. It has been shown (Pearson, '42) that intraperitoneal injection of a saline extract of less than 3 mg of submaxillary tissue of *Blarina* frequently is fatal to a 20-gm white mouse. It was also shown that mice injected with extract of approximately 15 and 30 mg of submaxillary-retrolingual tissue of *Sorex cinereus cinereus* were not seriously affected. I have now given white mice intraperitoneal injections of the following amounts of saline extracts of the submaxillary glands of several species of shrews: *Sorex pacificus sonomae,* 5.5, 7.4, 8.2; *Sorex palustris navigator,* 5.3, 11.3, 13.6; *Sorex trowbridgii montereyensis,* 4.3, 5.3; *Sorex vagrans vagrans,* 2.7, 3.5, 10.3; *Sorex sinuosus,* 6.7, 14.3, 20.0, 26.9; and *Cryptotis parva,* 3.9, 14.9. The dosages are given in milligrams of fresh shrew submaxillary tissue per 20 gm of mouse. For the first 4 species circumstances did not permit the use of fresh glands, so in these cases the glands of freshly killed animals were placed in acetone, removed a few days later, dried, and stored in a dried condition until use. Such treatment does not seriously alter the toxicity of *Blarina* glands if a large volume of pure acetone is used. The dosages for these 4 species are given, nevertheless, in milligrams of wet weight of tissue. I am indebted to Mr. Ernest P. Walker of the National Zoological Park in Washington, D. C., for the *Cryptotis* used in these experiments.

Only one mouse was killed by these injections despite the fact that dosages in many cases were considerably greater

than the lethal dose of *Blarina* extract. This one mouse died 8 hours after the injection of 10.3 mg/20 gm of extract of *Sorex vagrans*. Because of the long time elapsing between injection and death, and because the convulsions and protruding eyes produced by *Blarina* toxin were not manifest, I feel that *S. vagrans* does not have a poison comparable to that of *Blarina*. The other species tested must also be considered relatively non-toxic. The only effects noted were labored breathing in some cases for a few minutes after the injection and occasional hiccuping.

Protein digestion. Schmidt ('31) and Lawrence ('45) have suggested that shrew saliva contains a protein-digesting enzyme. I have tested for the presence of such an enzyme in the following manner. Four fresh submaxillary glands of *Sorex sinuosus* were ground with sand in 2.5 cm^3 of distilled water. Into this mixture was put a short length of 1-mm capillary tubing into which eggwhite had been drawn and coagulated by submergence in boiling water. Even after several days at room temperature no appreciable dissolving of the eggwhite in the tube could be discerned. Submaxillary glands of *Blarina brevicauda* preserved in acetone, dried, then prepared as described above also failed to demonstrate any protein digestion under these conditions.

Relative immunity of Blarina to its own poison. In a single test, 0.6 cm^3 of extract containing 28 mg of *Blarina* submaxillary tissue was injected intraperitoneally into another *Blarina* weighing about 20 gm. For two hours after the injection the animal appeared uncomfortable, the lips unusually pale, the respiration frequently jerky. By the next morning the animal seemed to have recovered, but it died a day and one-half after the injection. Death may have resulted as much from injection of a large volume of unsterile solution as from any salivary poison. One-third this amount of submaxillary poison injected into a mouse would have killed it in less than two hours.

CONCLUSIONS

It is clear that none of the species of shrews tested possesses a poison comparable in strength to that of *Blarina*. Since many of them have the curious salivary segment previously noted in *Blarina*, there is some doubt that this segment is the source of the poison in *Blarina*. The segment is better developed in *Blarina* than in the other shrews, but it is prominent enough in *Sorex* and *Cryptotis* for one to expect definite evidence of poison were this segment responsible for poison production. Why the segment of granular cells was not demonstrable in *Sorex cinereus* and *S. bendirii* is not clear. No apparent distinction of habits or phylogeny separates these two species from the others that possess the segment.

SUMMARY

The unusual segments of granule-filled cells of the submaxillary tubules of *Neomys* and *Blarina* are also present in the following shrews: *Sorex fumeus, S. pacificus, S. sinuosus, S. trowbridgii, S. vagrans*, and *Cryptotis parva*. They could not be demonstrated in *Sorex cinereus, S. bendirii*, or in the shrew-mole (*Neurotrichus gibbsii*). In two of 22 *Blarina* studied the granular segment was not found. These were the only estrous females sectioned.

No poison comparable to that of *Blarina* was found in submaxillary glands of *Sorex pacificus, S. palustris, S. sinuosus, S. trowbridgii, S. vagrans*, or *Cryptotis parva*.

Blarina is relatively immune to its own poison.

Extract of submaxillary glands of *Sorex sinuosus* and *Blarina brevicauda* showed no ability to digest protein when tested on eggwhite.

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PLATE 1

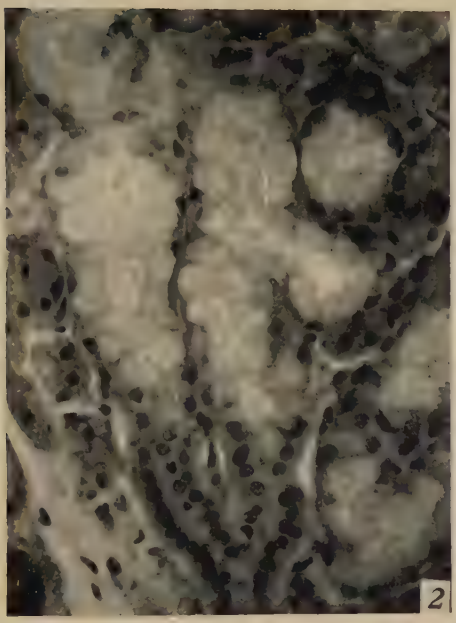
EXPLANATION OF FIGURES

2 Longitudinal section through two submaxillary tubules of *Blarina brevicauda.* Formalin fixation, hematoxylin and eosin stain. Note the enlarged, pale, granule-filled cells with shrunken nuclei. × 380.

3 Longitudinal section through two submaxillary tubules of *Blarina brevicauda.* Granules large and darkly stained following Zenker-formol fixation and Mallory's stain. × 650.

4 Longitudinal section through a submaxillary tubule of *Sorex palustris* showing a pale, granule-filled segment similar to that in figure 2 but less highly developed and with the nuclei of the granule-filled cells not shrunken. Bouin's fixation, hematoxylin and eosin stain. × 380.

5 Longitudinal section through a submaxillary tubule of *Sorex pacificus.* Zenker-formol fixation, Mallory's stain. The dark-staining granules are smaller than those of *Blarina* shown in figure 3. × 650.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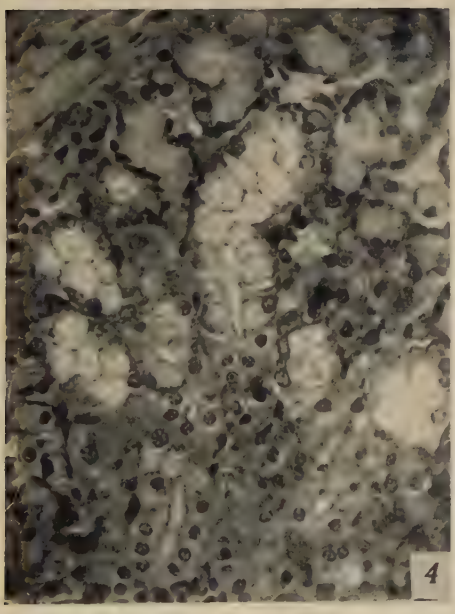
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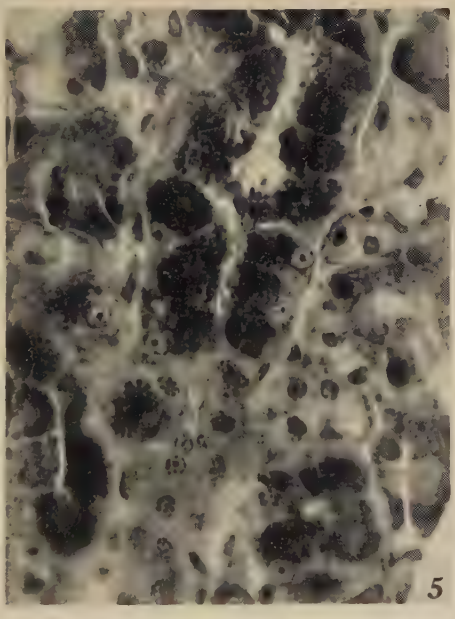
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ANOMALOUS VERTEBRAE FROM THE LUMBO-SACRAL COLUMN OF MAN

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THREE FIGURES

Anomalies of the lumbosacral vertebral column of man are legion and apparently were in the ancients (Manners-Smith, '09) even as they are today (Brailsford, '29). Indeed, they are so numerous and varied that at times it is only with difficulty that the normal can be separated from the abnormal. Although some of these variations occur with surprising frequency, others are quite rare. The purpose of this communication is to describe three specimens with the same unusual variation which were found in the course of examining the lumbosacral columns of the Terry Anatomical Collection.

In his description of the vertebral column of a young Egyptian female, thought to have been buried at about 600 to 500 B.C., Barclay-Smith ('11) presented a line drawing indicating that the 4th lumbar vertebra of this unusual specimen had a separation in the continuity of the neural arch between the superior articular process and pedicle on the right side. Both the line drawing and description of this vertebra make it clear that the loss of continuity was actually anterior to the superior articular process and not in the pars interarticularis where separations are relatively common (Rowe and Roche, '50).

Other accounts of specific cases presenting this particular defect have not been found in the literature, although Willis ('23) in his discussion of anomalies of the vertebral column presented a diagram which indicated by lines the regions in which the continuity of the neural arch may be interrupted

and includes the region between the superior articular process and pedicle as one. The same diagram was repeated by Brailsford ('29) and Willis ('31) but no illustrative photographs or cases were presented to show the actual vertebrae with separations between the superior articular process and pedicle. It is assumed, therefore, from the lack of descriptions in the literature and from the low incidence of such vertebrae in the Terry collection that such variations must be quite uncommon.

The three adult specimens to be reported in this communication were discovered in the course of examining 1539 lumbosacral columns consisting of 512 White males, 562 Negro males, 311 Negro females and 154 White females. In each of the three cases the defect involved vertebrae of the lumbosacral joint, and in each case more than one anomaly existed in the lumbosacral column. All three defects occurred in Negroes.

The first specimen, no. 864, was the vertebral column of a 42-year-old Negro male. There were 24 presacral segments, the 24th of which (lumbar 5) presented a loss of continuity between the left superior articular process and the pedicle. As can be seen in figure 1, the left superior articular process of the 5th lumbar vertebra was deformed and the corresponding inferior articular process of the 4th lumbar vertebra was very poorly developed presenting a concave, irregular articular surface into which the superior articular process of the last presacral vertebra projected. A small process of similar form and position as the mammillary processes of the upper lumbar vertebrae was present immediately lateral to the line of discontinuity, and the accessory and transverse processes of the involved segment were typical in morphology and position. The sacrum of this specimen presented a thin linear separation between the superior articular process and the lateral mass on the left side as well as failure of the inferior articular processes and arch of the first sacral segment to fuse with the articular processes and arch of the second sacral segment. This tendency for

the first sacral segment to be free from the remainder of the sacrum was also manifested anteriorly where the body was widely separated from the second segment, but not laterally, for there was solid fusion between the lateral masses of the two segments. Including the lumbarized first sacral segment, and excluding the first segment of the coccyx which is fused to the last sacral segment, the sacrum consisted

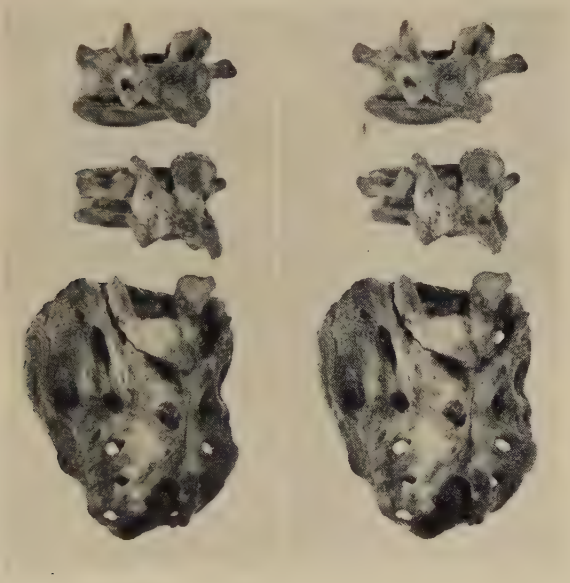

Fig. 1 Stereographic photographs of lumbar vertebrae 4 and 5 and sacrum from specimen no. 864, (Terry Anatomical Collection) Negro male, age 42. (Since proper appreciation of shape is obtained only by three dimensional viewing, this figure and the subsequent two are presented in stereographic photographs. The most satisfactory stereographic image is obtained by holding a stiff paper as a partition between the two figures and observing them so that it is possible to see only the right image with the right eye and the left image with the left eye. Make sure that each picture is illuminated equally. When viewed thus at approximately 10 inches, two images are seen at first, but if one gazes into the distance through the images they move together and fuse into one which is then seen in three dimensions. At first attempt the fused images are apt to fly apart when accommodation for detailed study is attempted because of the convergence which normally accompanies accommodation, but subsequent attempts become progressively more successful. Figures approximate one-third size.)

of 5 segments. A considerable asymmetry between the lateral masses of the two sides of the sacrum was also apparent, and from the position of the two auricular surfaces, as well as from the asymmetry of its superior articular processes it is very probable that the long axis of the sacrum was at a considerable angle to that of the rest of the vertebral column. A concurrent asymmetry of the innominate bones was apparent tending to confirm the above impressions of sacral angulation. Osteoarthritic changes about the joints of the region suggested that abnormal stress and strain may have been present during life.

The second specimen, no. 913, was derived from a 27-year-old Negro female. The 5th lumbar vertebra, which was the 24th and last presacral segment, revealed absence of the inferior articular process on the right side, and in its place a tiny projection of bone ending in a small, irregular, non-articular tip. In addition the lamina and isthmus of this vertebra were considerably narrower on the right side than on the left (see fig. 2). The first sacral neural arch presented a wide spina bifida with considerable loss of bone from the medial end of the ununited left lamina combined on the right side with the absence of any connecting bone between the superior articular process and lateral mass. Associated with the absence of the inferior articular process of L5 on this side, the right sacral superior articular process did not have an articular surface, and thus one-half of the first sacral neural arch was free, except for a rudimentary articulation with the superior articular process of the second sacral segment on the right. No other anomalies were present in the lumbosacral region and osteoarthritic changes were not remarkable.

The 5th lumbar vertebra and sacrum of the third specimen, no. 1521, a 54-year-old Negro male, are shown in figure 3. There were 24 presacral segments, the last of which was the 5th lumbar. The sacrum presented 5 segments, the first of which was involved by a spina bifida defect accompanied by a loss of continuity between the superior articular process

and the lateral mass on the left. As a result of these defects the left lamina, superior and inferior articular process and the spinous process of this vertebra constituted a separate portion which articulated with the inferior articular process

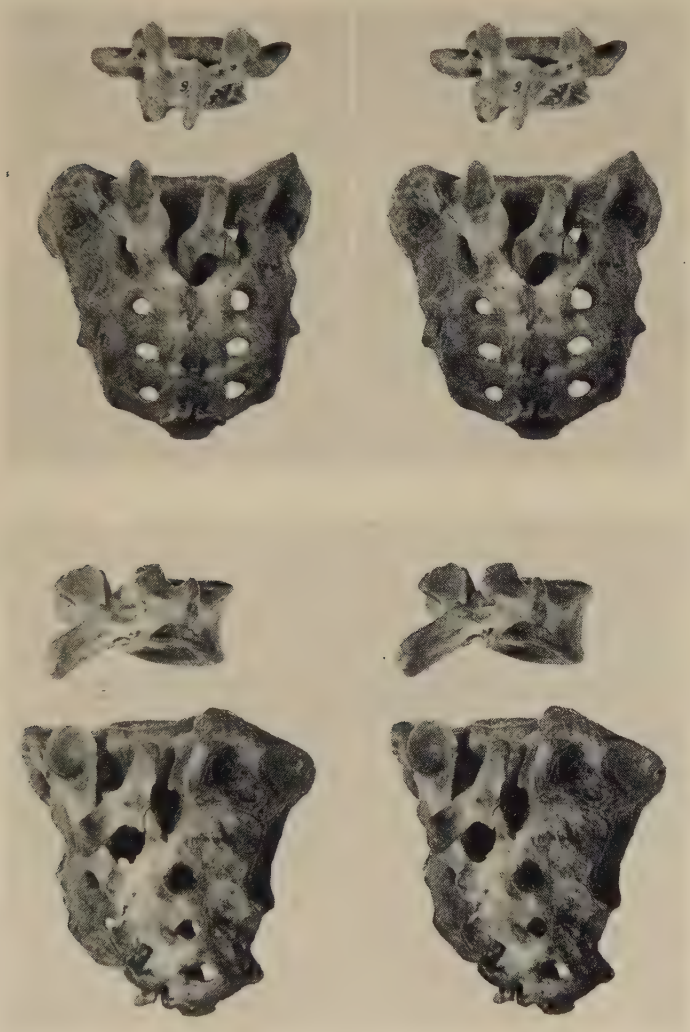

Fig. 2 Stereographic photographs of last lumbar vertebra and sacrum of specimen no. 913, (Terry Anatomical Collection) Negro female, age 27.

of the 5th lumbar vertebra and the superior articular process of the second sacral segment. There was considerable asymmetry of the inferior articular processes of the last lumbar vertebra because of a rudimentary left inferior articular process. An additional small articulation was formed on the

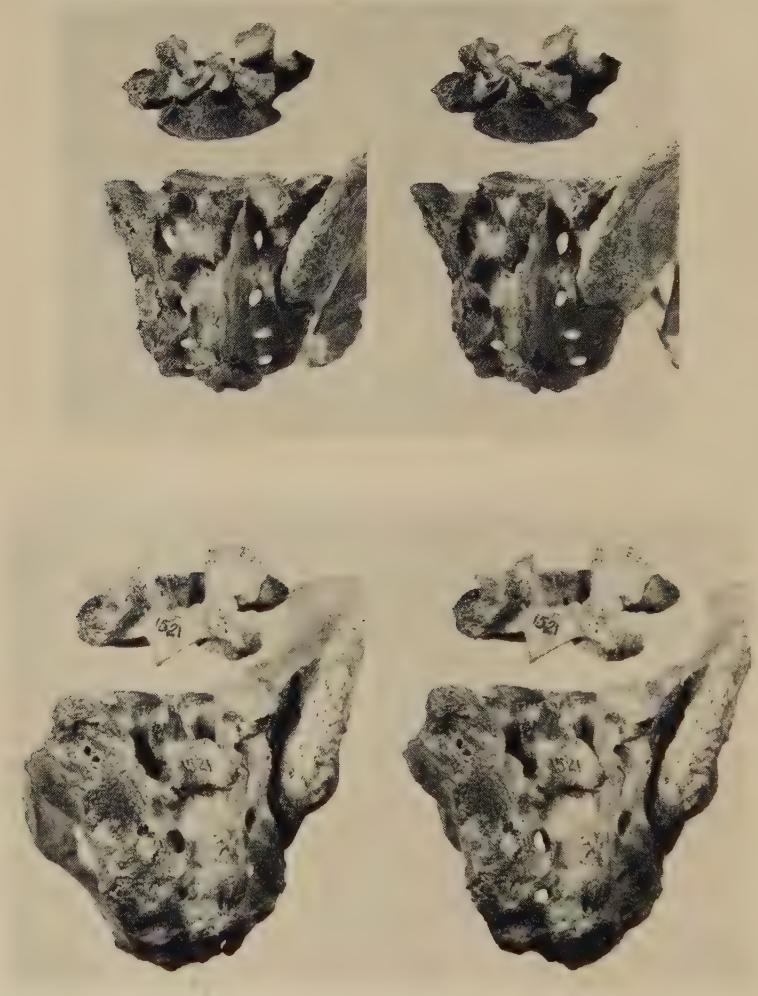

Fig. 3 Stereographic photographs of 5th lumbar vertebra and sacrum of specimen no. 1521, (Terry Anatomical Collection) Negro male, age 54.

left between the transverse process of the last lumbar and the lateral mass of the first sacral vertebra. A gross irregularity of the inferior surface of the body of the last lumbar vertebra corresponded to projections on the superior surface of the body of the first sacral segment (see fig. 3). On the right side the sacroiliac articulation was fused.

DISCUSSION

It has been shown many times that the normal pattern of ossification of the neural arch is for one ossification center to appear in the cartilage between the superior and inferior articular process of each half of the neural arch and to spread from there posteriorly to meet in the spinous process with the center for the opposite side, and anteriorly to meet at the neurocentral synchondrosis with the center for the body (Bardeen, '05; Batts, '39; Chandler, '31; Friberg, '39; Hitchcock, '40; Mall, '06; Noback, '44; Willis, '31). The separated fragment described here for one-half of the sacral neural arch could be explained easily, then, as failure of the ossifying process in one-half of the arch to fuse with that of the opposite side posteriorly and failure to reach the ossifying centrum or lateral mass anteriorly. In specimen no. 864 the first sacral arch apparently underwent fusion posteriorly in the usual fashion but no connection was established anteriorly on the left. However, the explanation of the loss of continuity found in the arch of the 5th lumbar vertebra of specimen no. 864 (fig. 1) is not so simple. In order for such a separation to occur as a result of anomalous development, the center of ossification for the involved half of the neural arch must have been divided near its point of origin or two centers for this side must have arisen independently. Descriptions of such division or independent origin of the centers of ossification for this portion of a lumbar vertebra have not been found in the literature. It is thought better, therefore, to leave unexplained that which is not understood for, although such speculations are easily made, proof for them, thus far, has not been obtained.

The rudimentary right inferior articular process presented by the 5th lumbar vertebra of specimen no. 913 may well be the result of failure of normal development of the ossification center for the inferior articular process. A center for the inferior articular process has been found to be separate from that for the rest of the neural arch once by Batts ('39) in a series of 200 consecutive observations of human fetuses and once (two anomalous centers in the same specimen) by Roche and Rowe ('50) in the examination of 73 fetal and newborn specimens. If such variation as total separation of its point of origin from the rest of the ossification center to which it normally belongs has been found, it seems reasonable to assume that such alteration in the process of ossification as to produce the above described variation might well occur.

SUMMARY

1. Three specimens are described in which there was a loss of continuity between the superior articular process and the pedicle of the first sacral segment. In two of these sacra spina bifida involved the same neural arch and thus one-half of the arch was free of any bony connection.

2. One case of unilateral rudimentary or absent right inferior articular process of the 5th lumbar vertebra is presented.

3. One case of separation between the lamina and mammillary process of the 5th lumbar vertebra is described.

4. The probable embryological basis for these variations is discussed.

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THE USE OF DENTAL ACRYLIC IN THE PREPARATION OF PRESSURE INJECTION CASTS OF THE AUDITORY APPARATUS AND PARANASAL SINUSES

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For many years Woods metal casts of the auditory apparatus and paranasal sinuses have served for study and teaching purposes. Preparation of these casts is expensive and time-consuming. A rapid and economical method of preparing these casts with dental acrylic is presented.

The technic of injecting the middle and internal ear with low melting point metal (Woods) and freeing the metal cast from the bone by corrosion processes was described by Siebenmann (1882). Siebenmann (1890, 1897) made extensive use of this technic in his classic studies on the pneumatization of the temporal bone. Casts prepared in this manner are valuable for teaching and research purposes. However, Woods metal is affected by strongly alkaline and acid solutions, therefore decalcification must be done in weak solutions over a period of many weeks.

Jones ('35), Guild ('35), Anson et al. ('38) and Bast and Anson ('49) have expressed doubt that all of the smaller air cells become filled when molten metal is poured into the bone and they have relied more extensively upon the wax plate reconstruction of stained serial sections. This method is accurate but very laborious.

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In order to overcome some of the disadvantages of preparations made with low melting point metals, a simplified technic of pressure injecting plastic substances into the temporal bone was developed. The principles of the process have been previously described (Seliger, Dolowitz and Swinyard, '48). Gray ('48, '49) described a technic of plastic infiltration of the temporal bone. This procedure requires 6 weeks for plastic impregnation and in our hands the results were found to be somewhat erratic. Young ('49) described a pressure injection technic for molten lead and Woods metal. The pressure equipment used is expensive.

Casts prepared by pressure injection of acrylic substances, according to the following technic, can be completed within a 4-hour period with negligible outlay for equipment and materials.

EQUIPMENT AND SUPPLIES REQUIRED

1 lb. Dental acrylic — Powdered monomer methyl methacrylate.[2]

4 oz. Liquid polymer.[2]

10 lbs. Dental plaster.

1 Galvanized iron pipe nipple or electrical conduit nipple $3\frac{1}{2}$ inches in diameter and 4 or 5 inches long.

2 $3\frac{1}{2}$-inch electrical junction box lock nuts or two $\frac{1}{2}$-inch segments of a $3\frac{1}{2}$-inch galvanized iron pipe coupling.

2 4-inch electrical switch box covers.

1 Metal rod $\frac{1}{4}$ inch in diameter and 1 inch long, which is threaded or welded into the center of one of the switch box covers.

1 Autoclave or any household type pressure cooker.

In our initial experiments the temporal bone was embedded in plaster in a Pryor dental injection flask using a Pryor piston cylinder for pressures. Since the injector flask costs nearly $40.00 this was abandoned and the readily available materials previously listed which can be obtained from any electrical or plumbing shop were used. The acrylic and plaster are avail-

[2] Nutone. Generously supplied by Floyd L. Burr, Manufacturer, San Francisco, California.

able at dental supply houses. Except for the autoclave or pressure cooker the equipment and supplies necessary to prepare about 20 casts can be obtained for approximately $5.00.

The pipe or conduit nipple (outside threaded) is cut into two longitudinal segments. The lock nuts or segments of pipe coupling (inside threaded) are screwed on the outside of the two segments of pipe nipple while they are held in apposition. The temporal bone is embedded in dental plaster within the pipe, therefore this arrangement enables one to remove the pipe quickly and easily from the plaster cylinder after polymerization has been completed.

PROCEDURE

The temporal bone is isolated from the remainder of the skull. The tympanic membrane and auditory ossicles should be removed through the external auditory meatus.

Soft beeswax is placed over the external auditory meatus. Powdered monomer and liquid polymer are mixed to a heavy putty consistency and the plastic is placed in the sigmoid sinus, jugular bulb and carotid canal. It can be squeezed into the internal auditory meatus and hiatus Fallopii with finger pressure. The cover with the metal rod attached is placed in position so that the tip of the rod is embedded in the beeswax of the external auditory meatus.

The bone is oriented in the pipe with the external auditory meatus directed upward and in the center of the pipe. Plaster is poured around the bone and in a few minutes is sufficiently set to hold the bone in position. The pipe is then completely filled with plaster and in about 30 minutes is set enough to allow the removal of the cover with the rod attached. The temporal bone is now completely embedded in plaster except for a channel one-fourth inch in diameter and one inch long which leads through the plaster to the beeswax which is in the external auditory meatus. The wax is removed from the meatus by inverting the plaster cylinder over a hot plate and allowing the melted wax to escape through the rod channel.

Plastic is then mixed to a heavy syrup consistency and poured into the opening leading to the external auditory meatus. It is often easier to use plastic of gelatinous consistency and roll out pencil-like pieces which are placed in the channel. When the opening is filled with plastic the rod on the cover is used to force the methacrylate into the meatus and all cavities of the middle and internal ear. As additional quantities of plastic are placed in the channel greater pressure becomes necessary to force it into the bone. If the packing is done on a low table the weight of the individual forcing the rod into the channel is adequate. If greater pressure is desired the rod can be forced into its channel with the use of a bench vise. When no further quantities of plastic can be forced into the bone, the pipe with the contained injected bone is placed in an autoclave or household pressure cooker and the methacrylate is polymerized for one and one-half hours at approximately 225°F.

After polymerization the lock nuts are removed, the pipe segments separated and the bone is easily removed from the plaster investment. Methyl methacrylate is remarkably resistant to acid and full strength hydrochloric acid can be used for decalcification. If the acid is boiled decalcification can be accomplished in approximately one hour.

Most of the casts reproduce with remarkable fidelity the tympanic cavity, tympanic antrum, mastoid air cells, smaller groups of pneumatic cells and all parts of the osseous labyrinth. The relationships of adjacent important structures are also shown. Casts prepared with this technic have reproduced virtually every group of air cells which have been described by the time consuming and laborious wax plate reconstruction method. Since comparison controls are not possible one cannot be certain that every minute pneumatic cell has been filled, however this simplified rapid technic could be utilized to supplement serial section reconstruction methods.

Medical students commonly encounter difficulty in understanding the orientation of the various components of the auditory apparatus and their relationship to important con-

tiguous structures. Usually huge papier-mâché models or a few sculptured temporal bones are the only aids available. Fragile casts of Woods metal made with considerable time expenditure cannot be made available indiscriminately. The plastic injection technic described can be applied to temporal bones taken from gross anatomy laboratory cadavers. Therefore, it was found desirable to request that students dissect only one temporal bone. The remaining bone can be cleaned in weak sodium hydroxide solution and used for the preparation of a cast as described. The department technician can easily prepare these and we have found them to be very useful adjuncts to teaching.

With the use of a larger sized container the authors have prepared accurate casts of the nasal cavities and paranasal sinuses.

SUMMARY

1. By means of pressure injection of methyl methacrylate, accurate and durable casts of the auditory apparatus can be produced rapidly and economically.

2. Filling the carotid canal, internal auditory meatus, sigmoid sinus and jugular bulb prior to injecting the auditory apparatus enables one to demonstrate the relationship of these structures to the middle and internal ear.

3. Casts can be prepared from either dried skull bones or cadaver bones and can therefore be made available in quantity for student use and are valuable teaching adjuncts for gross anatomy.

4. With slight modifications of the technic it is possible to make accurate casts of the nasal cavities and paranasal sinuses.

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POLYOVULY AND ANOVULAR FOLLICLES IN THE WILD NORWAY RAT[1]

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TWO FIGURES

A study of serial sections of wild Norway rat (*Rattus norvegicus*) ovaries done for other purposes (Davis and Hall, unpublished) showed incidentally the presence of follicles containing two ova and also structures which upon serial examination were found to be anovular follicles such as reported in the opossum (League and Hartman, '25). The purpose of this paper is to report on the occurrence of polyovuly and anovular follicles encountered in the course of this examination.

Hartman ('26) reviewed the literature on polyovuly in many species and reported its occurrence in the opossum. They are considered to be either lacking or very rare in the laboratory rat and have not been reported in the wild Norway.

Numerous serial section studies of ovaries have been made by various investigators in their investigation of the reproductive organs of the laboratory albino rat. Butcher ('27) examined serial sections of ovaries, discussed the origin of definitive ova in the white rat from birth to maturity but did not report on polyovuly. Lane ('40) studied 495 follicles in serial sections of ovaries from 17 pregnant rats. No men-

[1] Contribution from the Vertebrate Ecology Division of the Department of Parasitology of the Johns Hopkins University School of Hygiene and Public Health. This research was conducted under a grant from the International Health Division of the Rockefeller Foundation.

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tion of polyovuly was made. Lane ('38) described "aberrant follicles" in the immature rat, during examination of over 28,000 follicles in 100 ovaries from 60 rats ranging from 15–64 days in age and found polyovuly or polynuclei in 0.077% of the follicles. All such "abnormal follicles" were, with two exceptions, primary, and the author concluded that such structures were probably doomed to atresia rather than normal development. Cole ('36) studied experimental animals treated with mare gonadotropic hormone. He examined every 4th histologic section and reported on induction of superovulation, but apparently polyovuly did not occur. Marx ('41) studied the embryology and ovogenesis in the rat ovary giving a detailed account, in which appears one very interesting sentence: "In maturing females there are always a few ova undergoing cleavage in medium or large follicles and in one case a multiple follicle was found in an adult."

MATERIAL AND METHODS

Ovaries of 394 wild Norway rats captured in poultry warehouses in Baltimore were removed, fixed in Bouin's, and stained in toto with alum cochineal, then counterstained, after sectioning, with cotton blue. These 788 ovaries were examined to determine the number of generations of corpora lutea present as well as the number of corpora lutea in each. Thus, examination of the follicles was only casual and notation of polyovuly or polynuclei was made only incidentally.

OBSERVATIONS

Forty-seven, or 12% of the rats examined, had at least one polyovular follicle (fig. 1). This figure of prevalence is undoubtedly lower than the true value because cases in which the two ova may be separated and not appear in the same serial section would be easily overlooked in our study. Also, these observations were incidental to others that pre-occupied the attention in serial examination.

The relationship between polyovuly and fecundity was studied by comparing the number of new corpora lutea in

the ovaries with numbers of embryos found in the uterine horns of the same animal. More embryos than corpora lutea were encountered in 21% of the pregnant animals. Usually the excess was one or two embryos, though one individual exhibited an excess of 10 embryos, 23 embryos being found in its uterine horns. It was assumed in cases where more embryos were found than corpora lutea that transmigration

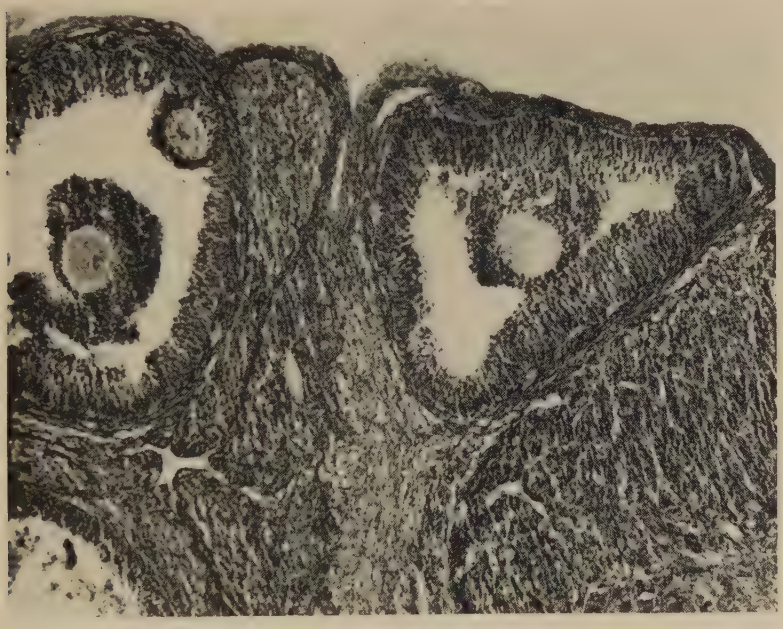

Fig. 1 Polyovuly in a follicle on the left. Normal follicle on the right. × 150.

of ova from one ovary to the opposite uterine horn did not occur. Transmigration does apparently occur in the rabbit (Brambell, '44) and sow (Corner, '21). The presence of a periovarial sac, which though not totally closed at all times is considered to be so during ovulation (Alden, '42), and the presence of two uteri with two ora cervices, seem to preclude the possibility of transmigration of ova in the rat.

It seemed possible that twinning after ovulation accounted for the excess of embryos. However, the dissection of 1084

embryos from their embryonic membranes failed to reveal a single case of twinning. A more likely explanation is that some follicles release more than one viable ovum. The general concensus of opinion seems to be that the polyovular follicles give rise to only one healthy ovum, the other ovum being doomed to atresia. Others contend that polyovular follicles do give rise to more than one individual (Brambell and Mills, '47; Allen, Brambell, and Mills, '47).

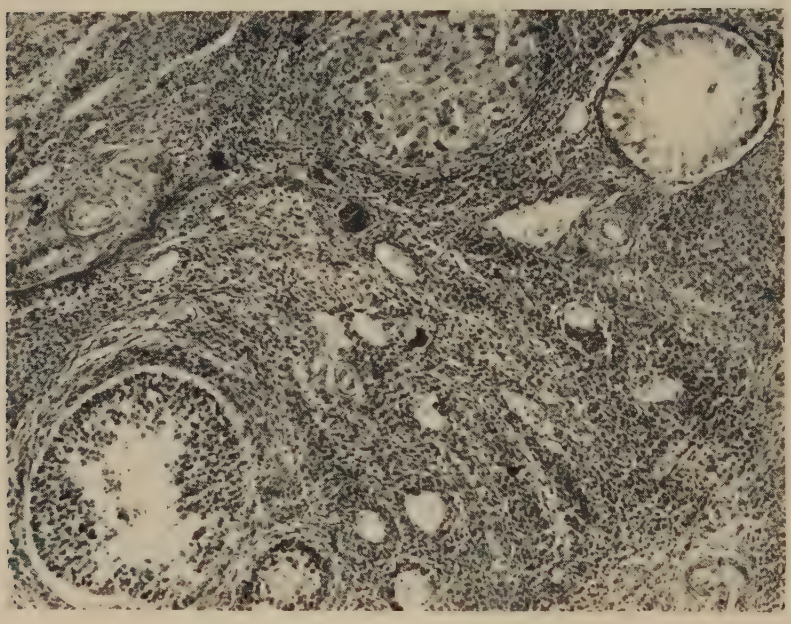

Fig. 2 Anovular follicle in upper right corner. Normal follicle in lower left corner. × 150.

In rats where the number of corpora lutea exceeded the number of embryos the discrepancy may be explained by several obvious possibilities: (1) Not all ova were fertilized. (2) Not all fertilized ova were successfully implanted, or if implanted, were resorbed. (3) Some of the structures counted as corpora lutea may have been luteinized follicles with trapped ova which therefore did not contribute viable ova.

Luteinization of follicles of all sizes in this species was found to be common and is to be reported elsewhere.

Certain ovaries also contained structures (fig. 2) which fit the description of "testis" cords as observed in the ovaries of the shrew by Wilcox and Mossman ('45). In serial sections these structures characteristically start as a blind end, continue for several times the length of a follicle of equal diameter in cross section and again terminate blindly. Histologically there is a well-defined basement membrane upon which rests a complex 2- or 3-layered epithelium consisting of high cuboidal or low columnar cells, and a "lumen" in the center. Much less frequently debris and even a well-defined though atretic ovum was seen in the "lumen." These structures apparently correspond to "anovular follicles" as described by League and Hartman ('25) and Barton ('45).

SUMMARY

The ovaries of 394 wild Norway rats (*Rattus norvegicus*) captured in poultry warehouses in Baltimore, Maryland, were sectioned serially. Histologic examination revealed that at least 12% of the rats exhibited polyovular follicles. It is suggested that the presence of more than one ovum in a follicle may account for the fact that 20 out of 95 pregnant rats had more embryos in the uterus than corpora lutea in the ovaries. Follicles without ova were also observed.

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TRANSPLANTATION OF HEMATOPOIETIC TISSUES INTO THE CIRCULATING BLOOD

I. EXPERIMENTS WITH LYMPH NODES IN NORMAL RABBITS[1]

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FOURTEEN FIGURES

INTRODUCTION

As has been pointed out by Korschelt ('31), transplantation in a broad sense includes blood transfusion although it deals with a fluid tissue, the blood, instead of a formed tissue. It is not strange therefore that several investigators have sought special advantages in the selective transfusion of suspensions of leucocytes or other wandering cells for experimental or morphological study, although transfusion was originally devised for clinical therapeutics.

Selective transfusions of leucocytes collected in great numbers from varied sources such as exudates, pus, blood, or blood forming tissues, were used in experiments as follows: polymorphonuclear leucocytes by Christeller and Eisner ('28), Wallbach ('29), Borghi ('30), Hanausek ('38), Osumi ('42), Farr ('46) and others; mononuclear cells by Hamazaki and Watanabe ('29); and lymphocytes by Erf ('40). In these experiments, leucocytes were vitally stained by such dyes as neutral red, Janus green, acriflavin hydrochloride, trypan blue, or lithium carmine in order to make it possible to follow the behavior of the transfused leucocytes in the blood or tissues of the recipients.

[1] The cost of this investigation has been defrayed by a grant from the Department of Education for scientific research.

According to Christeller and Eisner ('28), Borghi ('30), and Osumi ('42), however, transfused polymorphonuclear leucocytes degenerate too rapidly to exhibit any characteristic behavior. Erf ('40) was not able to ascertain the ultimate fate of transfused lymphocytes because they disappeared from the circulation within a short time. On the other hand, mononuclear cells, capable of storing colloidal dyes such as carmine, India ink, or trypan blue, are suitable for experiments of this type, because, if labelled vitally, they are easily traced in fixed and stained sections, and they survive for a much longer time than polymorphonuclears and lymphocytes and may even mutiply.

An interesting result has been obtained by Hamazaki and Watanabe ('29). These investigators collected a large amount of a heavy suspension of histiocytes containing carmine granules from the peritoneal exudate of rabbits which had been injected repeatedly with lithium carmine intraperitoneally, and administered this suspension into the ear vein or heart ventricle of a series of rabbits. The injected "carmine cells" showed a special affinity for the reticuloendothelial system, settled in this system, and took a shape similar to the reticuloendothelial cells. In the liver for example, they were fixed as stellate cells of Kupffer in great numbers. In addition, these "carmine cells" were still capable of ingesting India ink for several days.

These experiments with histiocytes suggest the possibility that other blood cells, especially immature forms, might also exhibit a characteristic behavior and that experiments of this type might contribute, from a new angle, to the solution of the unanswered problems concerning the blood cells and the blood forming tissues.

Accordingly, the author has endeavored for several years to elaborate a satisfactory new method for transplantation of hematopoietic tissues into the circulating blood. It is of course impossible to introduce these tissues directly into the circulation in the form of a coherent tissue. Their cellular constituents must first be freed from their stroma and pre-

pared in the form of a cell suspension in a physiologic saline solution or other liquid medium. Except for this preliminary procedure and the peculiarities of the circulating blood as an environment, there is no essential difference between this method and common transplantation. From the morphological point of view, therefore, the term transplantation is preferable to transfusion, and hence the former was adopted as the title of the present series of studies. This report, the first of a series on experiments made along this line, will deal with the transplantation of cells from the lymph nodes into the circulating blood.

MATERIAL AND METHOD

The animals used for transplantation were rabbits. In most cases the cells to be transplanted were collected from lymph nodes of 3 to 5 healthy rabbits about 6 months of age in the following manner: the large lymph nodes situated in the mesenterial roots were extirpated from every animal sacrificed, then carefully freed from fat, cut into as small pieces as possible in about 30 cm^3 of a physiologic saline solution, and the solution was gently stirred so that the cellular elements might be suspended in it. Thus a heavy suspension of cellular constituents of lymph nodes was obtained. The suspension was then filtered through a piece of sterilized double-folded gauze, washed once by centrifuging and filtered once more immediately before it was injected into another healthy rabbit. The great majority of the cellular elements of the suspension were lymphocytes of various sizes, while other types of cells were present only in a small percentage as shown in table 1.

The number of nucleated cells of the suspension, which is approximately equal to that of lymphocytes, was 100,000 to 150,000 per cubic millimeter; and at least one-half of these cells were demonstrated as alive when examined by supravital staining with Janus green and neutral red. In most cases, however, the suspensions were not stained prior

to the injection, because no fixatives are capable of fixing these dyes satisfactorily for study in tissue sections.

The whole amount of the suspensions, 20 to 30 cm^3, was administered as slowly as possible either into the ear vein or, under laparotomy, into the mesenteric vein of one of a series of healthy rabbits, ranging in weight 600 to 2,400 gm. The total number of injected lymphocytes per kilogram of body weight amounted to from 600 to 2,300 millions. The injection was confined to a single dose.

As control experiments, the same or greater amount of the lymph node suspension prepared in a similar manner was injected intravenously into one of another series of healthy rabbits, after its cellular constituents had been killed by freezing and thawing twice, or severely injured by heating at 50°C. for 30 minutes.

Differential leucocyte counts of the blood of the recipient animals were made at intervals of 15 to 60 minutes for the first 6 hours after the injection, and later at intervals of 12 to 24 hours. No blood examinations were made of animals which were injected in the mesenteric vein because laparotomy may affect the blood picture and confuse the results. Some animals were repeatedly injected with lithium carmine or India ink prior to the autopsy in order to check the behavior of reticuloendothelial cells.

At successive intervals after the single injection of lymph node suspension, the animals were killed by the injection of air, and the lung, liver, spleen, bone marrow, lymph nodes, and small and large intestines were studied in tissue sections, fixed in Zenker formol and stained with hematoxylin and eosin or hematoxylin-eosin-azure II.

EXPERIMENTAL RESULTS

1. Lymph node suspension injected into ear vein

The number of blood lymphocytes did not rise markedly, indeed less than had been expected, although an enormous number of lymphocytes was brought at a time into the cir-

TABLE 1

Differential cell counts (1000 cells) from wet smears [1] of the lymph node suspensions, fixed in Zenker formol and stained with hematoxylin-eosin-azure II

COUNT NO.	LYMPHOCYTES				NON-LYMPHOCYTIC CELLS				
	Small L.	Medium sized L.	Large L.	Lympho-blasts	Reticulo-endothels	Plasma cells	Pseudo-eosino-philes	Eosino-philes	Unclassi-fied cells [2]
1	922	40	16	3	2	6	6	3	2
2	785	177	31	4	0	0	0	1	2
3	904	77	8	6	0	0	0	1	4
4	940	37	6	10	3	1	0	3	0
5	858	61	23	47	5	0	2	4	0
6	934	45	13	2	0	0	0	1	5
7	884	74	10	27	0	3	2	0	0
8	865	95	13	11	0	4	4	1	7
9	916	47	14	17	1	0	0	1	4
10	893	66	17	17	4	0	0	1	2
Average	890.1	71.9	15.1	14.4	1.5	1.4	1.4	1.6	2.6
			991.5				8.5		

[1] The dry smear method was not used because it crushed most of the cells.

[2] Most of these cells resembled monoblasts.

culation. Erf ('40) observed in rabbits a transitory rise in the number of blood lymphocytes after intravenous administration and a return to the normal within several hours. According to Christeller and Eisner ('28), Borghi ('30) and Farr ('46), transfused polymorphonuclear leucocytes also disappear rapidly from the circulation and do not return.

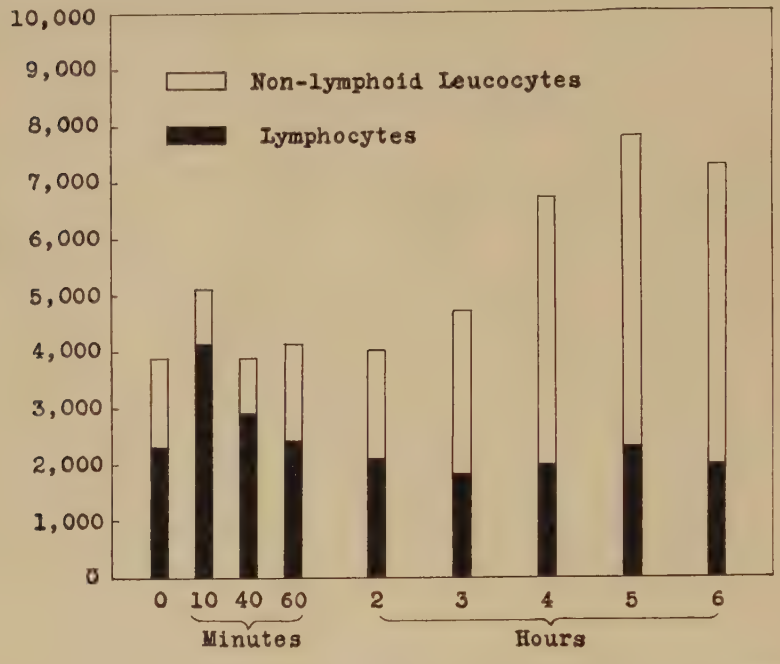

Fig. 1 Changes of the absolute number of lymphocytes and of non-lymphoid leucocytes of the blood immediately following the injection into the ear vein of the lymph node suspension. The total number of injected lymphocytes was 1,168 millions. Rabbit no. 6, 1,000 gm ♀.

It is, therefore, evident that the majority of injected lymphocytes have been eliminated from the circulation within two to three hours after injection (fig. 1).

A striking increase in lymphocytes occurs in the small blood vessels of the lung immediately after the injection. Especially in the capillaries of the alveolar wall, lymphocytes of larger types and nuclear debris, presumably derived from

the injected dead lymphocytes, were found in great numbers. In other organs, particularly in the liver and spleen, there was neither an abnormal increase in lymphocytes nor any indication of excessive degeneration of these cells during the first 24 hours after the injection. The major portion of injected lymphocytes therefore are arrested by the lung capillaries and thus removed from the circulation.

In the period later than 24 hours after injection, however, the circumstances were reversed. Lymphocytes in the lung were gradually decreased, but in the liver and spleen, they began to accumulate, first in the former and then somewhat later in the latter.

In the liver the chief accumulation of lymphocytes was in the interlobular periportal tissue spaces as numerous dense aggregations around interlobular branches of the bile duct and hepatic artery. These lymphocyte accumulations grew so rapidly that 48 to 72 hours after the injection almost all of the interlobular tissue spaces became occupied by lymphoid aggregations which were visible grossly as dark blue specks in stained sections (figs. 3 and 4). On the cut surface of the fixed liver, they appear as greyish streaks or nodules resembling the Malpighian bodies of the spleen. The results were strikingly uniform in all of the 6 examples (table 2).

In the intralobular sinusoids, during the same period, such a marked degree of accumulation of lymphocytes did not take place, although there was a slight increase in large lymphocytes and lymphoblasts scattered in the capillary lumen. In the liver, therefore, a focal accumulation of lymphocytes occurs in the interlobular tissue spaces. A similarly striking degree of focal accumulation of lymphocytes in these areas, so far as the writer is aware, is not seen except in lymphoid leukemia.

In the lumen of the radicles of the interlobular portal vein, before it empties into the capillary sinusoids, intravascular collections of lymphocytes often partially obstructed the lumen. They were attached closely to one side of the thin wall of the vein, adjacent to the periportal accumulations

of lymphocytes described above (fig. 5). The free surface bordering on the blood stream of such intravascular collections of lymphocytes was covered with a thin layer of fine fibrin threads. During the period from 48 to 72 hours after injection, a proliferation of endothelial cells from the neighboring endothelium spread along these fibrin threads, leading to the formation of new endothelium completely covering the free surface of these lymphocyte aggregations. At the same time the original, superfluous endothelial cells, no longer in direct contact with the blood stream, underwent

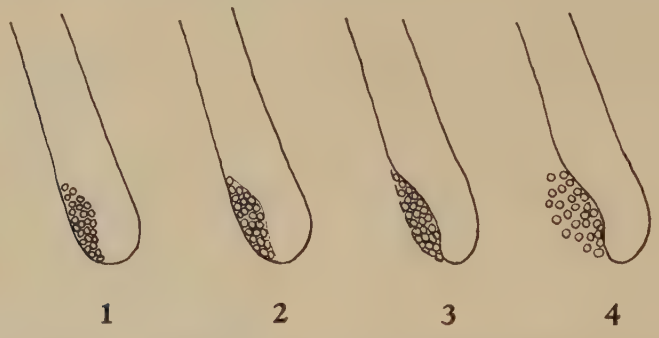

Fig. 2 Schematic illustration of the process of passive incorporation of lymphocytes from radicles of the interlobular portal vein into the periportal tissue spaces. 1. A naked lymphocyte collection attached closely to one side of the vessel wall nearest the periportal space. 2. The collection comes to be covered with fine fibrin threads. 3. The disintegration of the original endothelium (shown in dotted line) coincident with the formation, along the fibrin threads, of new endothelium covering the lymphocyte collection. 4. The final stage of the process.

atrophy and disintegrated gradually. As a consequence, the lymphocyte collections formerly found intravascularly came eventually to be situated outside of the vessel, merging with the neighboring lymphocyte accumulations of the periportal spaces. Since all intermediate stages of this process were seen in the sections of the liver, the mechanism of the passive incorporation of lymphocytes as represented schematically in figure 2 could readily be traced.

The predominating cells of the periportal accumulations of lymphocytes were of small and medium-sized types, among

which large lymphocytes and lymphoblasts were also mixed in comparatively large numbers as is the case in the lymph node (fig. 8). The admixture of large lymphocytes and lymphoblasts is a characteristic feature that distinguishes these lymphocyte accumulations from the inflammatory round cell infiltrations of the periportal spaces. Mitotic figures of these large lymphocytes and lymphoblasts were often seen and less frequently also those of the medium sized (fig. 9), and there was no indication of a rapid degeneration of the accumulated lymphocytes. It is therefore apparent that the lymphocytes accumulated in the liver tend more to proliferation than to degeneration. Occasionally, a few eosinophilic granulocytes occurred among the accumulated lymphocytes, but their occurrence was inconstant and therefore seems to be of minor significance. Pseudoeosinophilic granulocytes were usually not found. There was also no admixture of plasma cells, histiocytes and reticulum cells in the newly formed accumulations of lymphocytes in the periportal spaces.

The hepatic cells showed no significant changes except a variable degree of vacuolation of the cytoplasm which recovered rapidly. The stellate cells of Kupffer were often enlarged, some having two or more nuclei, others in mitoses, but there was no indication of extensive proliferation and migration into the interlobular tissue spaces such as, according to Dieterich ('27–'28) and Pagel ('39), might lead to the formation of round cell infiltrations. Phagocytosis of fragments of disintegrated lymphocytes by these cells was not seen as often as had been expected. In some instances, megakaryocytes of both mature and immature types occurred in the capillary sinusoids, usually accompanied by a few promyelocytes and myelocytes, but no evidence was obtained in favor of a direct transformation of the injected lymphocytes into these myeloid elements.

The third site of accumulation of lymphocytes was the spleen, which became two to several times its normal size. Histologically, there was a marked increase of the white pulp due to accumulations of lymphocytes in the perifollicular

regions. Neighboring follicles often became confluent (fig. 6). The follicles themselves, however, remained almost unaltered (fig. 7). Such accumulations of lymphocytes occurred somewhat later than those in the liver, usually beginning at the 48th hour and reaching the maximum at the 72nd hour (table 2).

The characteristic feature of the lymphocyte accumulations in the spleen was the presence of relatively great numbers of large lymphocytes and lymphoblasts with extraordinarily basophilic cytoplasm (figs. 7 and 10). They were often in mitosis, but more frequently their nuclei had undergone a degeneration through pyknosis or rhexis. Thus in the spleen, in sharp contrast with the liver, the accumulated lymphocytes tended more toward degeneration than proliferation.

In the red pulp, in contrast to the perifollicular areas, there was a slight increase in lymphocytes. The nuclear debris which is normally found in comparatively large amount, mostly phagocytosed by macrophages, was also increased to some extent. Megakaryocytes were met more frequently in the red pulp of the spleen than in the liver, usually accompanied by a few promyelocytes and myelocytes.

The focal accumulations of lymphocytes in the spleen, therefore, are in the perifollicular areas and correspond to those in the periportal spaces of the liver.

Among organs other than the liver and spleen, the bone marrow, and small and large intestines were studied with especial care, for these organs have been said to be the most important sites of the elimination of the blood lymphocytes (Bunting and Huston, '21; Jordan and Speidel, '23; Jordan, '36). So far as the present histological examinations are concerned, however, there was no indication of an extensive elimination of the injected lymphocytes by these organs. Lymphocytes found normally in the mucous membrane, especially between the epithelial cells of small and large intestines were not significantly increased by a single injection of the lymph node suspension. Likewise in the bone marrow there was neither any increase in lymphocytes nor the forma-

tion of lymphoid tissues. The most striking effect in the bone marrow was on the megakaryocytes; they became definitely phagocytic, often including several polymorphonuclear leucocytes at a time in their cell bodies.

Since it was expected that the lymphocytes accumulated in the liver might be transferred by the lymph flow and reach the regional lymph nodes, the hepatic, gastric and mesenteric lymph nodes were carefully studied. Histological examination gave no evidence of a loss of lymphocytes through the lymph stream.

In the blood of the recipients, there was always a severe leucopenia with a considerable reduction in granulocytes, during the period in which lymphocytes accumulated in the liver and spleen. During the same period the percentage of monocytes was always somewhat elevated.

2. Lymph node suspension injected into mesenteric vein

Since the major portion of the lymphocytes injected into the ear vein are arrested at first by the lung capillaries for a time and later accumulate in the liver and spleen, there is a possibility that many have been lost on the way to these two organs. If the injection is made into the portal vein, one might expect all the injected lymphocytes to reach the liver immediately without any loss on the way. Actually, in the present group of experiments half the number of lymphocytes was required for the same effect, as can be seen from a comparison of both groups of experiments in table 2.

After mesenteric vein injection, at first a marked increase in lymphocytes occurred in the capillary sinusoids and the portal vein, not in the periportal spaces. A considerable degree of accumulation of lymphocytes did not take place in the latter regions until the 24th hour. In other words, a period of time must elapse before an accumulation of lymphocytes occurs in the periportal spaces. It is worthy of notice that the same period of time is also necessary when the injection is made into the ear vein (table 2).

TABLE 2

The relation of the degree of accumulation of lymphocytes to the number of lymphocytes injected and the site of injection

SITE OF INJECTION	TIME AFTER INJECTION	RABBIT NUMBER, BODY WEIGHT AND SEX	LYMPHOCYTES INJECTED PER KG WEIGHT (IN MILLIONS)	LIVER: PERIPORTAL ACCUMULATIONS	SPLEEN: PERIFOLLICULAR ACCUMULATIONS
	12 hours	no. 25, 1,900 gm ♂	1,940	—	—
	24 hours	no. 24, 2,000 gm ♀	1,800	++	—
	48 hours	no. 13, 700 gm ♂	1,860	+++	+++
Ear	48 hours	no. 14, 650 gm ♂	1,350	++	++
vein	48 hours	no. 102, 1,400 gm ♀	1,600	+++	*
	72 hours	no. 38, 1,500 gm ♂	1,330	+++	++
	72 hours	no. 46, 2,400 gm ♂	1,250	+++	+++
	72 hours	no. 51, 1,400 gm ♀	2,300	+++	+++
	90 minutes	no. 29, 2,500 gm ♂	600	±	—
Mesen-	24 hours	no. 22, 2,200 gm ♀	1,000	++	—
teric	48 hours	no. 28, 2,700 gm ♂	640	+++	±
vein	48 hours	no. 32, 2,400 gm ♂	600	++	±
	72 hours	no. 31, 2,400 gm ♂	700	+++	++

The designation +++ means the highest degree of accumulation of lymphocytes, as seen in figures 3–4 and 6–7; ++ a somewhat lesser degree.

* The spleen was extirpated prior to the injection.

As seen in table 2, accumulations of lymphocytes were also produced in the perifollicular areas of the spleen, but always to a lesser extent than in the preceding group of experiments.

3. Subsequent behavior and fate of lymphocyte accumulations

Despite certain differences as noted above, the selective accumulation of lymphocytes in the periportal spaces of the liver as well as in the perifollicular regions of the spleen is produced essentially in a similar manner in both groups of experiments. It is thus apparent that the excess of lymphocytes produced by the injection of lymph node suspensions is removed from the blood stream and deposited in both areas. It should be noticed here that the accumulations in both organs are characterized by a comparatively abundant admixture of large lymphocytes and lymphoblasts, often in mitosis, just as in the lymphatic apparatus. Furthermore, it must also be taken into consideration that reticuloendothelial cells, although contained in an extremely low percentage, are also admixed in the injected lymph node suspensions. The question which arises, therefore, is whether these lymphocyte accumulations may give rise to genuine lymphatic apparatus. In order to answer this question, the subsequent fate of the lymphocyte accumulations of both the liver and spleen was traced in a series of rabbits for three weeks after the injection.

In the gross, there was a striking difference in behavior between the lymphocyte accumulations of the liver and those of the spleen. In the spleen, the lymphocytes accumulated in the perifollicular areas had already disintegrated to a large extent on the 5th day, being replaced entirely by connective tissue during the next several days. In the liver there was a marked tendency toward proliferation of the lymphocytes accumulated in the periportal spaces, often leading to the formation of nodules resembling lymphatic appartus with-

out germinal centers which are common in some lower vertebrates (Takawa, '43).

During the period from the 5th to 10th day, small accumulations of lymphocytes which were situated at the bifurcations of the portal vein or portions of large accumulations adjacent to the vessel wall grew and often projected markedly into the lumen of the vein (figs. 11, 13 and 14). By that time many such accumulations, their cellular constituents having been reorganized, had assumed the appearance of lymph nodules without germinal centers (figs. 13 and 14), in which, however, a network of argyrophil fibers had not yet fully developed. On the other hand, lymphocyte accumulations which were rather remote from the wall of the portal vein and therefore near the liver cords disappeared gradually, apparently through continuous degeneration of lymphocytes (fig. 12), and were eventually replaced by connective tissue. Occasionally, in the periportal spaces plasma cells occurred in comparatively large numbers during the course of degeneration of the accumulated lymphocytes.

The later behavior of the accumulations, however, was not as uniform as in the two earlier groups of experiments. In 3 of 6 examples there was no indication of any additional growth of the lymphocyte accumulations. Such inconstancy in the results appears to be due to some extent to constitutional differences in the recipient animals.

In three weeks after the injection, not only the lymphocyte accumulations but also the nodules were no longer seen in the places where they had occurred. They had been entirely replaced by connective tissue with a few lymphocytes and plasma cells. The spleen remained still markedly enlarged from a fibrosis of the perifollicular regions where lymphocytes had accumulated to a great extent.

4. Control experiments

In this group of experiments, the cellular elements of lymph node suspensions had been killed either by freezing and

thawing twice, or severely injured by heating at 50°C. for 30 minutes before injection. In both cases the results were strikingly uniform. No accumulations of lymphocytes such as had been seen in the preceding groups of experiments were produced either in the liver, spleen or other organs. It is therefore apparent that the injected dead or injured lymphocytes disintegrated rapidly in the blood or tissues of the recipient animals, and that the disintegration of these lymphocytes itself is not capable of inducing secondarily any local accumulation of lymphocytes in the definite areas of the liver and spleen. This was confirmed further by the finding that daily injections of 0.5 gm of sodium nucleinate prepared from cow lymph nodes, though administered for several days, did not cause any accumulations of lymphocytes in both organs.

The disintegration of injected lymphocytes proved to cause other effects on the recipient animals, however. In the first place, it brought about occasional occurrence of megakaryocytes in the liver and spleen, usually accompanied by a few myeloid elements, and secondly it elicited the phagocytic activity of the megakaryocyte of the bone marrow. On these effects I shall make a detailed description in another paper.

DISCUSSION

Whether the lymphocytes which accumulated in the interlobular spaces of the liver and in the perifollicular regions of the spleen were of external or internal origin must be considered. A number of facts indicate that they were derived from the injected lymph node suspensions rather than from the blood lymphocytes of the recipients.

(1) Control experiments with dead or injured lymphocytes failed to produce such accumulations in both areas; (2) relatively greater numbers of large lymphocytes and lymphoblasts, often in mitosis, were among the accumulated lymphocytes than are found in the circulating blood; (3) the difference between the behavior of the injected lymphocytes and of myeloid elements (Osogoe, '43) or of histiocytes

(Hamazaki and Watanabe, '29); and (4) the mechanism of emigration of the injected lymphocytes from the interlobular portal vein into the periportal spaces.

The majority of the lymphocytes injected into the ear vein are at first arrested by the lung capillaries as a result of mechanical regulation by these capillaries. Similarly, lymphocytes injected into the mesenteric vein by the intralobular capillary sinusoids of the liver. In the further behavior of the injected lymphocytes, it is important that a period of 24 to 48 hours must elapse before a considerable accumulation of lymphocytes occurs in the particular areas of the liver and spleen, even when suspensions are injected directly into the portal circulation. This fact suggests that the process is essentially no longer mechanical, but characteristic of the lymphocyte. This is emphasized by the finding (Osogoe, '43) that the cells of bone marrow transplanted into the circulating blood, after nearly the same latent period, heap up within the intralobular sinusoids instead of in the periportal spaces of the liver and either in the meshes of the reticular stroma of the red pulp or in the lumen of the venous sinuses instead of in the perifollicular regions of the spleen. According to Hamazaki and Watanabe ('29), transfused histiocytes have a selective affinity for the reticuloendothelial system. Comparison of the behavior of the lymphocyte with that of other types of cells indicates that the lymphocyte has a special affinity for the periportal spaces of the liver and the perifollicular regions of the spleen, and that the focal accumulation of lymphocytes in both areas is due to this affinity.

Of the routes by which the injected lymphocytes may be transferred to the periportal spaces of the liver, the first to be considered is the portal circulation. Injections of the suspensions directly into the portal vein were more effective in producing the periportal accumulations than were injections into the ear vein (table 2). The frequent occurrence of intravascular collections of lymphocytes in the portal vein, especially in the lumen of its radicles before emptying into the capillary sinusoids, favors this route. According to Wakim

and Mann ('42), in the mammalian liver, there are arteriovenous anastomoses between corresponding interlobular branches of the hepatic artery and those of the portal vein. From this it can easily be conjectured that the lymphocytes injected into the ear vein are transferred with the arterial blood from the lung and, passing through these arteriovenous anastomoses, finally arrive at the radicles of the interlobular portal vein, where they are detained before entering into the capillary sinusoids and form intravascular collections which are closely attached to the side of the vessel wall nearest the periportal spaces.

The further behavior of such intravascular collections of lymphocytes in the interlobular portal vein is of particular interest, for the mechanism of transfer of lymphocytes into the periportal spaces can readily be traced (fig. 2). Essentially, the process is a passive change of situation of the lymphocyte collections from inside to outside of the vessel by the formation of new endothelium covering the collections and the simultaneous disintegration of the original endothelial cells which are no longer in direct contact with the blood stream. Such passive incorporation alone, however, does not fully explain the mechanism of formation of the large accumulations visible grossly in the periportal spaces. Active migration of lymphocytes due to their own mobility, therefore, also plays an important role in the formation of periportal accumulations.

The present results are closely related to the problem of the normal fate of the lymphocyte. That more lymphocytes are lost daily from the blood stream than are present at any one time has been established by the quantitative studies on the output of lymphocytes through the lymph stream made by Rous ('08), Davis and Carlson ('09), Bunting and Huston ('21), Yoffey ('33), Reinhard ('45) and Adams, Saunders and Lawrence ('45), and others. The fact that the complete lymphatic blockade is followed by almost complete disappearance of lymphocytes from the blood stream (Blalock et al., '37) presents additional evidence.

The mechanism of elimination of lymphocytes from the circulation has recently been reviewed by Ehrich ('46). A favorite concept is that they migrate into the lumen of the intestinal tract and are excreted there (Bunting and Huston, '21). Erf ('40), however, believes that the gastrointestinal tract is more a source of new lymphocytes than a site of elimination for these cells, because gastroenterectomy upon rabbits had no marked effect on the elimination of hetergenic lymphocytes brought into the circulation, but caused from the beginning a severe and persistent lymphopenia. Jordan ('36, '39) and Kindred ('40), claim that lymphocytes produced in excess are filtered off in the bone marrow where as hemocytoblasts they become ancestors of the erythroid cells. Since in the present experiments the elimination of injected lymphocytes from the circulation took place neither through the intestinal tract nor in the bone marrow, but occurred to a great extent in the liver and spleen, more attention should be given to the latter regions as sites of elimination of lymphocytes from the circulation.

The present experiments, however, are of more significance in throwing light on the problem of the histogenesis of so-called "lymphoid metaplasia" than on the normal fate of the lymphocyte. As noted already, the lymphocyte accumulations produced in the periportal spaces of the liver are able to grow and resemble to some extent the lymphatic apparatus, and that such change can take place exclusively in close relation to the wall of the portal vein. The latter fact is of especial interest because, in certain lower vertebrates such as Aves, Reptilia and Anura, lymphatic apparatus is often found in the wall of veins, especially at bifurcations. Such lymphatic apparatus may be regarded as belonging to the venous system rather than to the ordinary lymphatic system (Takawa, '43). Therefore, some types of lymphoid metaplasia, as observed in the present experiments, may be explained from the phylogenetical point of view as a reawakening of such lymphatic apparatus of the venous system.

Some mention should be made of the common occurrence of a slight degree of lymphocyte infiltration in the periportal spaces of the liver, especially around interlobular branches of the bile duct. The degree of such lymphocyte infiltration may be increased markedly without inflammatory processes in the liver, for example, after the sensitization to heterogeneous proteins (Wallbach, '29; Pagel, '39; Osogoe, unpublished observations), x-ray irradiations (Horii, '49), and in association with the so-called "lymphatic constitution" (Kettler, '40). The periportal accumulations of lymphocytes in the present experiments and in lymphatic leukemia can be regarded as the extreme instances of this condition.

SUMMARY

1. A suspension of cellular constituents from the mesenteric lymph nodes of 3–5 adult rabbits was given in a single injection either into the ear vein or into the mesenteric vein of another healthy rabbit.

2. From 48th to 72nd hour thereafter, there occurred a striking focal accumulation of lymphocytes, including large lymphocytes and lymphoblasts, in the radicles of the interlobular portal vein, in the periportal spaces of the liver, and in the perifollicular regions of the spleen.

3. This did not occur in other organs except for a transitory, diffuse accumulation of these cells in the small blood vessels in the lung immediately after injection into the ear vein.

4. Injections into mesenteric vein were more effective than those into the ear vein in producing these effects, especially in the periportal spaces of the liver.

5. The incorporation of accumulations of lymphocytes from the interlobular portal vein into the periportal spaces was observed.

6. The lymphocyte accumulations produced in the liver showed a tendency toward growth and became like lymphatic nodules without germinal centers, in the close vicinity of

the wall of the portal vein; in the spleen they underwent degeneration.

7. When the cellular elements of the suspension were killed or severely injured prior to injection, no accumulations of lymphocytes were produced anywhere.

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EXPLANATION OF PLATES

All of the photomicrographs except figures 3, 4 and 6 were taken from preparations, fixed in Zenker formol and stained with hematoxylin and eosin.

PLATE 1

EXPLANATION OF FIGURES

3 Frozen section of formalin-fixed liver (lobus dexter) showing a large periportal accumulation of lymphocytes. Forty-eight hours after the injection into the mesenteric vein. × 80.

4 Gross appearance of the same section shown in figure 3. The periportal accumulations of lymphocytes are seen as numerous dark specks. × 4.

5 Section through the liver showing an intravascular collection of lymphocytes in the lumen of a radicle of the interlobular portal vein. Notice fine fibrin threads covering the free surface of the lymphocyte collection. Some lymphocytes of the collection are emigrating through the thin wall of that vein into the periportal space where a small accumulation of lymphocytes has already been formed. Forty-eight hours after the injection into the ear vein. × 400.

6 Gross appearance of the cut surface of a formalin-fixed spleen. Notice a marked increase of the white pulp. Seventy-two hours after the injection into the ear vein. × 5.

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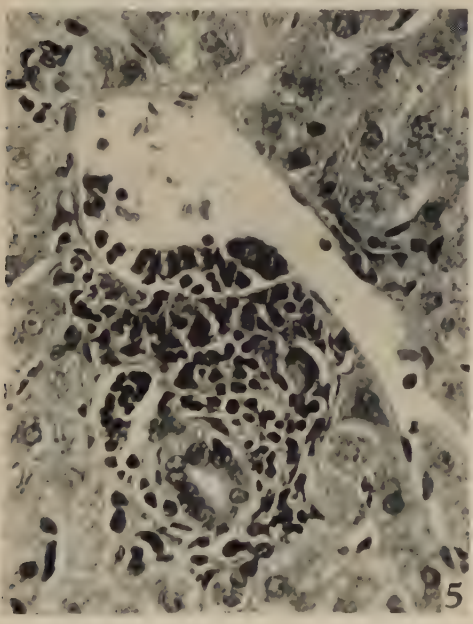

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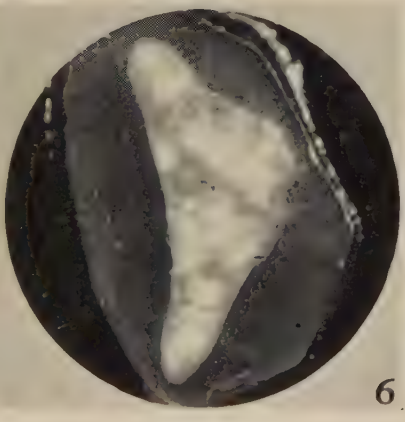

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PLATE 2

EXPLANATION OF FIGURES

7 Section from the same spleen shown grossly in figure 6, illustrating accumulations of lymphocytes of larger types in the perifollicular region. Seventy-two hours after the injection into the ear vein. × 100.

8 A small accumulation of lymphocytes in the periportal space of the liver including a large lymphocyte (to the right from the center) and a lymphoblast (to the left from the center). × 1000.

9 Mitosis (Mt) in a large cell, probably a lymphoblast, in a small periportal accumulation of lymphocytes. To the right and above the mitosis, amitosis (Amt) in a medium-sized lymphocyte can be seen. × 1000.

10 High power magnification of the large lymphocytes accumulated in the perifollicular region of the spleen shown in figure 7. The accumulated lymphocytes show extraordinarily strong basophilia and assume irreguar shape, one of which, however, is in mitosis (Mt). × 1000.

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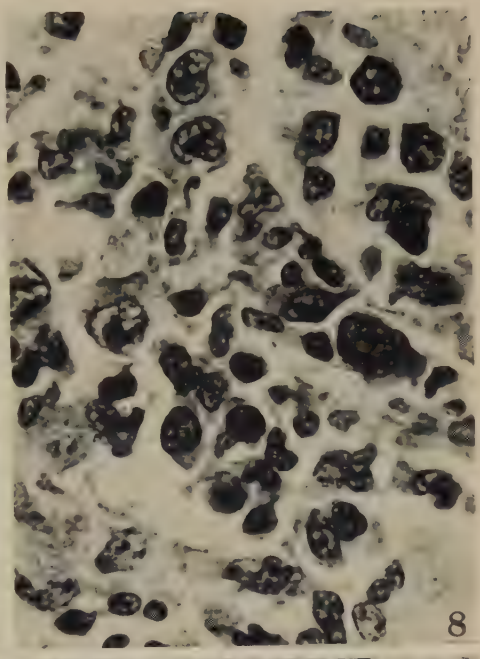
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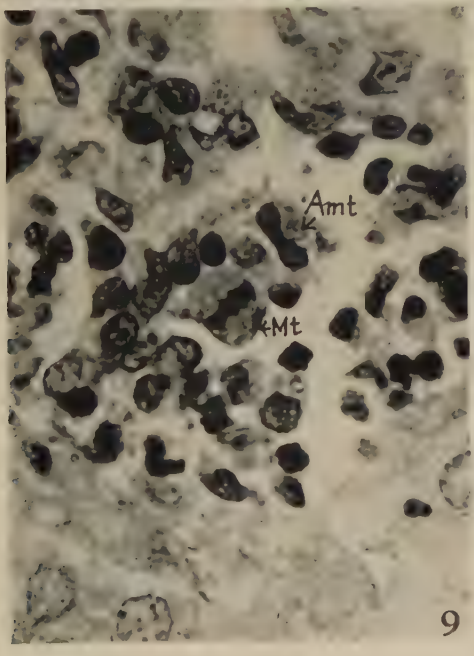
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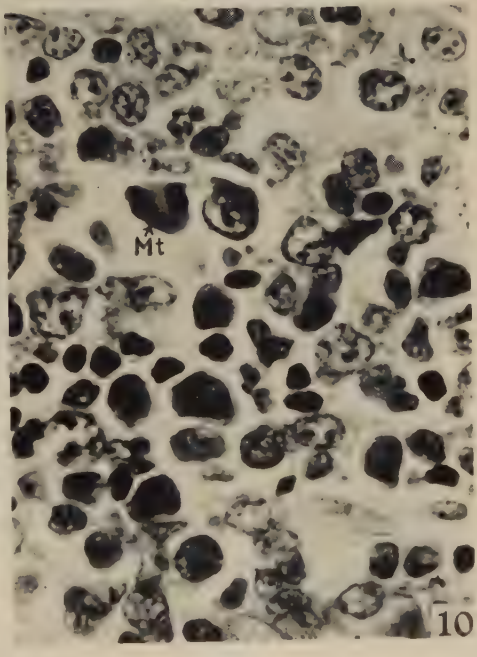
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PLATE 3

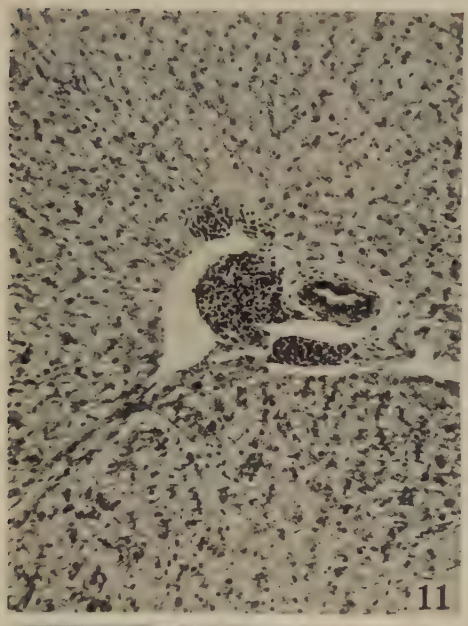
EXPLANATION OF FIGURES

11 A growing, small accumulation of lymphocytes in the bifurcation of the interlobular portal vein of the liver. Seven days after injection into the ear vein. ×100.

12 Section from the same liver illustrated in figure 11 showing a large lymphocyte accumulation of the periportal space. The major portion of the accumulation shows degenerative changes except its left lower portion nearest the portal vein having a narrow lumen. × 100.

13 Nodule-like accumulations of lymphocytes formed along the interlobular portal vein of the liver. Ten days after the injection into the ear vein. × 100.

14 High power magnification of a nodule seen in the center of figure 13. × 400.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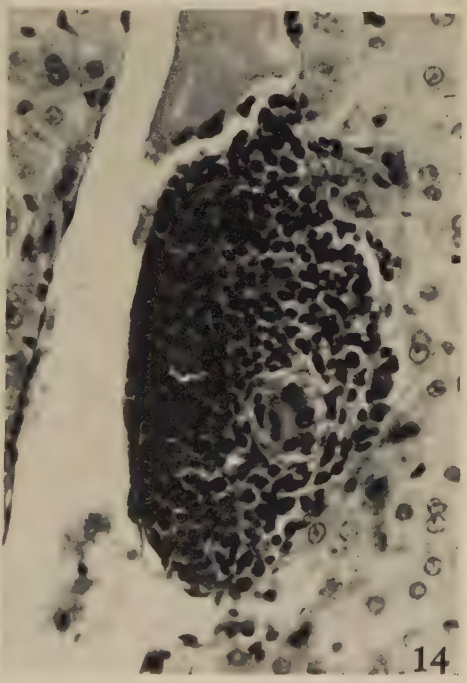
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fertility, and physical activity were noted. The work performed by the intestine has been divided into two phases — digestion and absorption. Anatomically the intestine is divided into two parts, large and small, but the work is shared jointly although not equally by the two parts. Theoretically a diet may affect structurally the whole intestine, or one of its parts.

Experimental studies on the effect of diet upon the intestine of fish, amphibia, and birds have been reported by Yung (1883, '09), Gulland (1898), Lonnberg ('02), Weiss ('01), Houssay ('02), Babàk ('06), Roux ('06), Schepelman ('06), and Elven ('28). These studies sprung primarily from the

[1] This investigation was supported by a grant from the Faculty Research Committee of the University of Pennsylvania.

observations of de Cuvier and Milne-Edwards that the intestine was longer in herbivorous than in carnivorous animals. In addition, search for proof for Lamarck's theory of "use and disuse" was stimulating morphological investigation.

Another interest in the problem developed later when advances in the medical sciences acted as a stimulus to research in the relationship of diet and growth. In addition, the establishment of the value and importance of vitamins in the diet was of the greatest assistance in interpreting the conflicting findings in earlier investigations.

Wetzel ('28) studied the relation of the weight and length of the intestine to the body in rats which had been fed plant, meat, and mixed diets. The plant and mixed diets were fed to three, the meat diet to 7 rats. The plant diet consisted of wheat (sometimes corn) supplemented occasionally with oats, rye, barley, fresh fruits, nuts, and germinated peas. The meat diet consisted of raw beef alternated with raw horse meat, including the bones; occasionally raw frog muscle was supplied. Fresh milk was given daily. The mixed diet was a combination of the meat and plant diets. He found that the large intestine was heavier on the plant diet than on either the meat or the mixed diet, while the small intestine was lighter than on either of the other two diets. In the rats on the mixed diet both the small and large intestine were intermediate in weight.

Addis ('32) reported measurements on the intestine of rats which had been fed a concentrated diet or a contrasting bulky diet which contained 40% dry alfalfa and 10% dry agar by weight, for 44 weeks. He found a 48% increase in the weight of the large intestine, and a 10% increase in the weight of the small intestine on the bulky compared with the weight on the concentrated diet. These percentages were based on the area of the body surface. The reported increase on the bulky diet is, however, not to be interpreted as a real increase because the body surface on the bulky diet was considerably less than the body surface on the concentrated diet.

Wierda ('42) reported that rats which had been fed diets containing raw liver had a longer small intestine than rats which were fed a meat-free diet. However, rats which were fed raw beef or cooked dried beef showed no significant increase in the length of the small intestine. It was also reported that the length of the colon was not increased by the addition of meat to the diet. Rats which had been fed a diet containing from 10 to 30% finely ground agar showed no increase in the length of the small intestine; however, the caecum and colon were definitely longer than in the concentrated diet rats. The surface area of both small (excluding the villi) and large intestine was greater on the agar and meat diet than on the concentrated diet but the surface of the large intestine was definitely greater on the agar diet. The mucosa of the small intestine showed a definite response to the meat diet, the villi being large, high, wide and thick, and differing in these respects from the villi on the balanced concentrated diet.

Localized overgrowths of the wall of the colon were found in rats on a high-fat diet (Wierda, '43). The great size of the overgrowths which assumed the shape of diverticula indicated the existence of an exceeedingly high growth potentiality. A similar finding was reported by Carlson and Hoelzel ('49) using a bulky diet.

MATERIALS AND METHODS

In these experiments 50 albino rats, including an equal number of each sex (Wistar strain) were maintained for 44 weeks on 4 different diets beginning at the time of weaning (see table 1). The animals were housed in screen-bottom cages and both food and water were kept continuously before the animals. The agar was finely ground, so that neither it nor any of the other ingredients could be separated from the diet by the rats. The salt was a modification of the Osborne-Mendel salt mixture (Wesson, '32). The rats used were the offspring of 10 matings, and each litter was spread over the 4 dietary groups as far and as evenly as possible so that

any hereditary differences would be distributed equally. At the end of the feeding period the rats were deprived of food for 24 hours before they were asphyxiated with coal-gas and autopsied. Each rat was weighed and its intestine carefully detached, slit open, and washed free from its contents. It was

TABLE 1

Average of measurments, with probable error, of 50 rats after 44 weeks of experimental feeding

NO. OF ANIMALS	COMPOSITION OF DIET		GROSS BODY WEIGHT	WEIGHT OF TOTAL FAT	WEIGHT OF SMALL INTESTINE	WEIGHT OF LARGE INTESTINE
14	Balanced		257 gm	16 gm	3.21 gm	1.83 gm
	Concentrated		± 10	± 1.9	± 0.08	± 0.04
	Casein	18%				
	Lard	23				
	Cornstarch	53				
	Salt mixture	4				
12	Bulky		261 gm	29 gm	3.76 gm	3.31 gm
	Casein	18%	± 11	± 1.4	± 0.21	± 0.17
	Lard	23				
	Cornstarch	23				
	Agar	30				
	Salt mixture	4				
12	Carbohydrate		268 gm	29 gm	4.26 gm	1.85 gm
	Casein	14%	± 12	± 6.8	± 0.24	± 0.06
	Cornstarch	83				
	Salt mixture	4				
12	Fat		278 gm	41 gm	3.86 gm	1.74 gm
	Casein	25%	± 11	± 5.2	± 0.15	± 0.07
	Lard	69				
	Salt mixture	6				

The diet of each rat was supplemented daily with 5 drops of cod liver oil and 0.4 gm of dry yeast.

then dried lightly with filter paper, placed in a stoppered weighing bottle, and weighed. The kidney was also carefully removed, dried by blotting with filter paper, and weighed in a stoppered weighing bottle. Finally the whole carcass was analyzed for fat using a modification of the Rose-Gottlieb

method for fat determination. The method consisted of saponification of each rat separately with potassium hydroxide, after which the volume was brought up to two liters, using a calibrated volumetric flask. An aliquot (10 cm^3) was transferred to a Röhrig tube and the fat separated by washing three times with 25 and 15 cm^3 of ether and petroleum ether. The combined washings were then freed from ether by evaporation on a steam bath to a constant weight. From this value the total amount of fat in the rat was calculated.

RESULTS

The averages of the measurements taken are shown in table 1. The average body weight at autopsy was nearly the same on the 4 diets. The greatest variation was between the balanced concentrated diet (257 gm) and the fat diet (278 gm). The gross weight on the bulky and concentrated diets was intermediate — 261 gm on the bulky diet and 268 gm on the carbohydrate diet. When the significance of these differences was tested statistically by the method of the probable error of the difference of the mean, it was found that there was no significant difference in gross weight. The probability was only equal that a difference in weight existed between the rats on the balanced concentrated diet that weighed the least and the fat diet rats that weighed the most.

The average amount of fat in the body (cellular and extracellular) at autopsy was least on the balance concentrated diet (16 gm) and greatest on the fat diet (41 gm), while on both the bulky and the carbohydrate diets the amount was 29 gm. When these figures were tested for validity by the method of the probable error of the difference of the mean it was found that it was a practical certainty that the fat diet rats possessed a greater amount of fat than the balanced concentrated diet rats. There was no significance to the difference in the amount of fat in the rats on the other diets.

The weight of the body of the rats, exclusive of fat, ranged less widely than the gross weight. The range of the net weight was from 241 gm on the balanced concentrated diet

to 232 gm on the bulky diet. In body tissues which were not saponifiable there was a spread of only 4% among the 4 groups.

The small intestine was heaviest on the carbohydrate diet, and lightest on the balanced concentrated diet. The difference in weight was 32%. The weight of the small intestine was on the average nearly the same on the fat diet and the bulky diet, and was not significantly less than on the carbohydrate diet, nor greater than on the balanced concentrated diet. The average weight of the small intestine on the 4 diets was 3.77 gm. On the balanced concentrated diet the weight of the small intestine was 17% less than the average of all 4 dietary groups; on the carbohydrate diet it was 11% greater; on the fat diet it was 2% greater, and on the bulky diet it was very nearly identical with the average. When these differences were checked by the probable error of the difference of the mean it was found that the probability was high that the small intestine was heavier on the carbohydrate than on the balanced concentrated diet. The probability was low for a real difference in weight between the other groups.

The large intestine was considerably heavier on the bulky diet than on the three contrasting (concentrated) diets. When the absolute weights were compared, it was found that the large intestine was 78% heavier on the bulky diet than on the carbohydrate diet; 80% heavier than on the balanced concentrated diet; and 90% heavier than on the fat diet. The difference between the weight of the large intestine on the bulky diet and on the three contrasting diets was great enough to make it highly probable that the large intestine was heavier on the bulky than on any of the other diets. When this test for significance was applied to the differences in weight of the large intestine on the carbohydrate, balanced concentrated, and fat diets, it was found that the probability was low that a real difference existed.

The average weight of the kidneys was greatest on the carbohydrate diet and least on the bulky diet. The difference in weight between these two groups was 27%, a difference

which when tested statistically indicated a high probability of a real difference. The average weight of the kidneys on the balanced concentrated diet was 17% less than on the carbohydrate diet, and on the fat diet the kidneys weighed 11% less than on the carbohydrate diet. But the kidneys of the balanced concentrated, fat, and carbohydrate diet animals were not significantly different in weight.

Table 2 shows the average weight of the kidneys in grams, and the ratio of the weight of the small and large intestine to the kidney weight after feeding the experimental diets for 44 weeks. On the balanced concentrated diet the small intestine was 155% of the weight of the kidneys; on the bulky

TABLE 2

The average weight of the kidneys in grams, with probable error; and the ratio of the weight of the small and large intestine to kidney weight after feeding the experimental diets for 44 weeks

TYPE OF DIET	KIDNEY WEIGHT	SMALL INTESTINE	LARGE INTESTINE
Balanced concentrated	2.07 ± 0.07	1.55	0.88
Bulky	1.91 ± 0.08	1.96	1.73
Carbohydrate	2.44 ± 0.10	1.74	0.75
Fat	2.19 ± 0.09	1.76	0.79

diet 196%; and on the carbohydrate and fat diet 174% and 176% respectively. In terms of kidney weight the small intestine was on the average 26% heavier on the bulky diet than on the balanced concentrated diet; and 12% heavier than on either the carbohydrate or fat diet.

The large intestine on the bulky diet was 173% heavier than the kidneys; on the balanced concentrated diet 88% heavier; on the carbohydrate diet 75% heavier; and on the fat diet 79% heavier. In terms of kidney weight the large intestine was 96% heavier on the bulky than on the balanced concentrated diet; 130% heavier than on the carbohydrate diet; and 118% heavier than on the fat diet.

DISCUSSION

The purpose of this experiment was to determine the effect of indigestible material in the diet upon the weight of the small and large intestine. Four diets were employed, one of which was a bulky diet containing a relatively large amount of agar — a poorly digestible substance, and three were contrasting, concentrated diets which contained no such ingredient but varied in respect to materials furnishing the calories. Of the three concentrated diets the fat diet had the highest caloric value — 7.21 calories per gram; the carbohydrate diet 3.88; and the balanced concentrated and bulky diets each had 4.91. To satisfy the caloric requirement it was necessary for the rats on the bulky diet to eat 4 times more material than the rats on the fat diet because dry agar is very bulky and has a low caloric value. Furthermore after the agar becomes wet it swells so that actually the intestine of the bulky diet animals handled approximately 10 times more material than the intestine of the rats on the fat diet. The carbohydrate and balanced concentrated diets did not swell upon wetting. Nevertheless, 4.5 times more material was handled by the intestine on the carbohydrate, and 5 times more on the balanced concentrated than on the fat diet. Wetzel ('28) did not calculate the relative amount of material handled by the intestine of his experimental animals, but Addis ('32) estimated that the intestine of his bulky diet rats handled 4 times more material (by volume) than the intestine of the "smooth" (concentrated) diet rats. Wetzel dealt particularly with the problem of the effect of meat, and of plant diets upon the weight and length of segments of the intestine, as well as the whole tract.

Both Wetzel ('28) and Addis ('32) encountered difficulty in interpreting the observed weight of the intestine on the contrasted diets because of the difference in body size of the rats. In Wetzel's experiments the difference was not great, but he believed that it accounted for the relatively heavier small intestine on the "plant" diet compared with the "flesh" diet. In the experiments of Addis the body weight of the

bulky diet rats was not much more than half that of the "smooth" (concentrated) diet animals, consequently no direct comparison of the weight of the intestine was made, but its weight was compared with the body surface. (Using the formula — body surface $= 11.3 \times 0.666$ body weight.)

In the present experiments the rats weighed approximately the same, at autopsy, on the 4 diets. This fact indicates that agar can compose as much as 30% of the diet of the rat without a resultant decrease in the growth rate of the body compared with the artificial diets containing no bulky ingredient. The fat determination shows that only the fat diet rats contained significantly more fat than the bodies of the leanest, i.e., the concentrated diet rats. The weight of the non-fat part of the rats was nearly the same on each of the diets, a fact which shows further that the potentiality for growth was not inhibited by any of the diets.

At autopsy the rats which had been fed the carbohydrate diet had a small intestine which exceeded the average weight on the other diets, and was also significantly heavier (statistically) than in the rats which had been fed the balanced concentrated diet. It is not clearly seen why there is this contrast in effect between these two diets. According to the theory of Roux ('06) a concentrated diet, rich in easily digestible substances, is capable of stimulating the growth of the small intestine, thereby enabling a greater and more rapid digestion and absorption of the food, but in these experiments the bulky diet (30% agar) rats showed a heavier small intestine, at autopsy, than the rats which had been fed a concentrated balanced diet. Such a finding is plainly not in accord with Roux's theory — for the balanced concentrated diet is clearly more easily digested than the bulky diet. According to Wetzel ('28) and Wierda ('42) the feeding of a meat diet or a diet containing meat resulted in a longer and heavier small intestine in rats than a plant diet, or a diet containing casein as the source of protein. Addis ('32), however, reported finding an 8% increase in the weight of the small intestine on a bulky diet, but since this calculation is

based on the body surface of rats which weighed only half as much as the controls, there is reason to doubt the existence of a real increase.

The weight of the large intestine at autopsy clearly shows that hypertrophy has occurred on the bulky diet. For although the body size was nearly the same as on the concentrated diets, the large intestine was 78% heavier than on the carbohydrate diet; 80% heavier than on the balanced concentrated diet; and 90% heavier than on the fat diet. These values are considerably higher than Addis ('32) found. He reported only a 48% increase in weight for the large intestine but the contrast in his diets was less than in these experiments and, in addition, his bulky diet animals were only half the size of those on the concentrated diet.

The weights of the kidneys were compared with the weight of the small and large intestine because the kidneys have been used frequently as a criterion for enlargement of other organs, although it has been shown by several investigators that the coefficient of correlation of kidney weight with body weight is very high. In the rat on a stock diet values of 0.96 and 0.95 were reported for males and females respectively (Webster, Liljegren and Zimmers, '47). In terms of kidney weight the small intestine was found to be heaviest on the bulky diet, exceeding the weight on the concentrated balanced diet by 26% and both the carbohydrate and fat diets by 12%. The large intestine was likewise heaviest on the bulky diet in terms of kidney weight, exceeding the weight on the balanced concentrated diet by 96%, and on carbohydrate and fat diet by 130 and 118% respectively. The observed weight of the large intestine after 44 weeks of feeding a bulky diet indicates an enlargement of approximately the same magnitude as Flint ('12) observed in dogs from whom 50 to 70% of the small intestine had been removed surgically. No gross changes in the large intestine, such as diverticula, were observed in any of these rats but there was a marked increase in caliber (diameter) as well as in the thickness of the wall.

In aged rats hypertrophy of the colon through the development of diverticula has been reported by Wierda ('43) and Carlson and Hoelzel ('49).

SUMMARY

A group of 50 albino rats were fed an artificial unbalanced diet beginning at the time of weaning and continuing for 44 weeks, to determine the effect upon the weight of the small and large intestine. The animals were asphyxiated with coal-gas after having been starved for 24 hours. The small and large intestine and the kidneys were then carefully isolated, trimmed free from attached tissue, and weighed using glass stoppered weighing bottles to prevent dehydration. The carcass was saponified and the amount of fat in it was determined by a modification of Rose-Gottlieb method for fat determination. The weight of the small and large intestine on the 4 diets were then compared with each other and with the body (gross, and minus fat) and kidney weight.

The gross weight of the rats was found to be not significantly different on the 4 diets; however, those which had received the fat diet possessed a significantly greater amount of fat than those which had received the balanced concentrated diet. The net weight of the body (gross weight minus fat) was even more nearly the same on the 4 diets than the gross weight.

The small intestine was found to be heaviest on the carbohydrate diet and lightest on the balanced concentrated diet. This difference was great enough to be statistically significant; the average difference was 32%. The small intestine was intermediate in weight between these two extremes on the fat and bulky diets. These differences in weight cannot be accounted for on the basis of the concentration of the diet. The heavier weight of the intestine on the carbohydrate diet may be due to the ease with which the diet is digested, and the rapidity with which it moves through the small intestine, but the balanced concentrated diet differs hardly enough from the carbohydrate diet to account for the lightness in weight of

the small intestine on the balanced concentrated diet. The large intestine was significantly heavier in the rats which had been fed the bulky diet than in those which had received the concentrated diets. The average weight was 78% greater than on the carbohydrate diet; 80% greater than on the balanced concentrated diet; and 90% greater than on the fat diet.

In terms of kidney weight the small intestine was on the average 26% heavier on the bulky diet than on the balanced concentrated diet, and 12% heavier than on either the carbohydrate or fat diet. The large intestine was, in terms of kidney weight, 96% heavier on the bulky diet than on the balanced concentrated diet; 130% heavier than on the carbohydrate diet; and 118% heavier than on the fat diet. The increased weight of the colon was not due to diverticula, but primarily to an increase in diameter, and a thickening of the wall.

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A NOTE ON THE CAROTID BODY AND CAROTID SINUS OF VARANUS MONITOR

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THREE FIGURES

The present work was prompted by Boyd's ('42) study of the nerve supply of the branchial arch arteries in *Vipera berus*, and it was thought advisable to study the innervation and characteristics of this region in other reptiles also. The first to be studied was Varanus and incidentally it revealed many interesting features which throw light on the probable evolution of the carotid sinus-carotid gland complex.

MATERIAL AND METHODS

Six adult Varanus monitor were dissected providing 12 specimens for study. The terminal part of the common carotid artery along with the commencement of the internal and external carotid arteries was removed in one piece. The specimens were then washed free of blood in .6% NaCl solution. Six specimens were fixed in Bouin's fluid, cut serially and stained with haematoxylin and eosin. The remaining 6 specimens, also sectioned serially, were stained by the Bielschowsky's technique.

OBSERVATIONS AND COMMENTS

Gross examination of the fresh specimens showed an elongated fusiform swelling, pinkish in colour, on the dorsal side of the arteries, extending from near the termination of the common carotid artery, involving the bifurcation, and continuing up the internal carotid artery for a variable distance.

On an average the fusiform area was 7.2 mm long and 3.4 mm at its greatest diameter.

Examination of serial sections showed that the lumen of the vessel was significantly dilated at the termination of the common carotid artery, the dilatation extending up to and involving the commencement of the internal carotid artery.

The lumen of the vessel was uniform in outline, there being no irregularities as are present in Ophidia (Boyd, '42) or emerald lizard (Palme, '34). The most significant feature of the sections, however, was the presence of a large mass of epithelioid cells on the dorsal aspect of the vessel, localised in the region of the carotid sinus. The presence of epithelioid cells in the walls of the branchial arteries has been described both by Boyd ('42) and Palme ('34), but they have not mentioned any regular arrangement of these cells. In Varanus monitor, on the other hand, we find that the greater part of the fusiform swelling, seen on naked eye examination, is made up of an aggregation of these cells on the dorsal wall. These cells lie enmassed between the adventitia and the media of the vessel, and from the latter fibromuscular trabeculae extend outwards and break up this cellular mass into separate loculi. Each smaller region thus marked out is occupied by closely packed columns of epithelioid cells about 5 to 8 rows in thickness. These cells are more or less polygonal in shape and take up the eosin stain rather faintly. They possess a very distinct, centrally placed, round nucleus, with a well marked nucleolus in each.

Through the middle of each such loculus there runs a blood sinusoid filled with the characteristic nucleated red blood cells. These blood sinusoids at places open into the lumen of the carotid artery and their endothelium is directly continuous with that of the sinus region of the artery.

Figure 1 is a microphotograph of a transverse section across the bifurcation of the common carotid artery under the low power of the microscope. It shows the aggregation of epithelioid cells with fibromuscular trabeculae passing between the columns of cells.

The nerve supply of this area is from the glossopharyngeal nerve by a twig which passes caudally in between the internal and external carotid arteries and enters the portion of the media which separates the epithelioid cell mass from the lumen of the carotid sinus. The nerve rapidly breaks up into

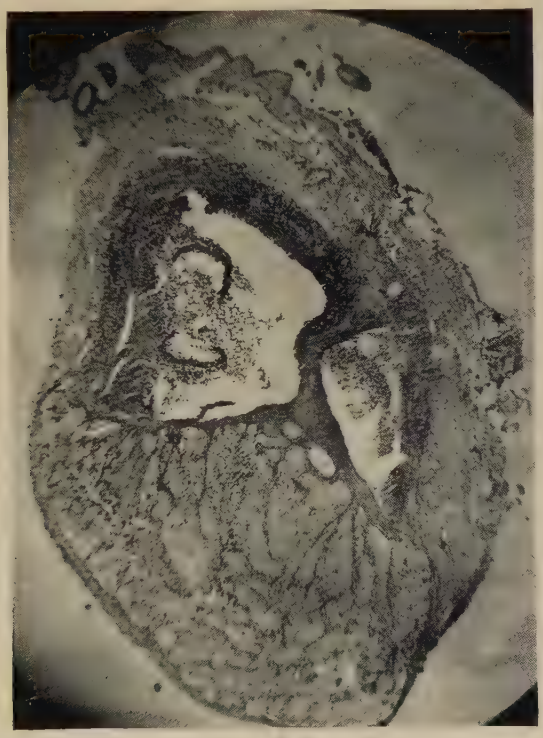

Fig. 1 Transverse section across carotid gland of Varanus monitor, stained with haematoxylin and eosin. The two arteries seen in section are the internal and external carotid arteries. The epithelioid cell mass is present on one side of the bifurcation.

a number of fine filaments which go partly to supply the media of the arterial wall and partly to the epithelioid cell mass. As in Ophidia the fibres terminating in the wall of the vessel are only slightly branched. The nerve fibres destined for the epithelioid cell mass pass along the fibromuscular trabeculae and finally, breaking up into finer filaments end among the

cells. Figure 2 shows nerve fibres passing in one such trabecula in the midst of epithelioid cells.

It is worthy to note that no pigment cells of any kind are present in this region in Varanus monitor in contradistinction to the descriptions in Ophidia and Amphibia.

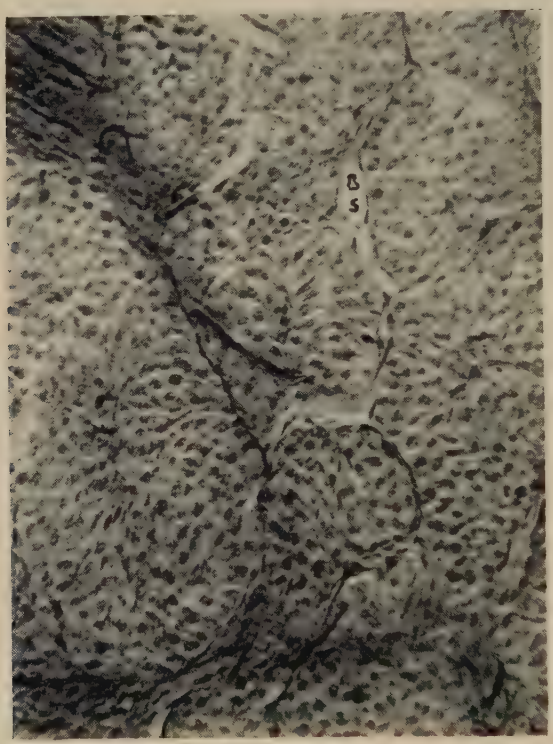

Fig. 2 High power microphotograph of epithelioid cells of carotid gland of Varanus monitor (Bielschowsky's silver impregnation), showing nerve fibres traversing the cell mass. At BS is a blood sinusoid.

The nature of the so-called epithelioid cells has been discussed at length by Boyd ('42): "Until more definite evidence on the precise origin and histogenesis of these cells is available it seems more satisfactory to use a non-committal term for them. Glomus cells or epithelioid cells are, at present, more satisfactory as terms of reference for them than para-

ganglionic cells." Again, Boyd states: "Leaving as undetermined, then, the nature of the cells described by Trinci, Palme and Adams in the neighbourhood of the commencement of the reptilian internal carotid artery, the presence of the thickening in the wall of the artery, and of the nerve endings in and around this thickening, enables one to state that the reptilian third arch arteries have a specialised nervous apparatus associated with them . . ."

According to the above argument the structure in Varanus monitor has evolved even more since (a) the lumen of the carotid sinus region is quite uniform in outline, (b) the adventitial coat is not as markedly thickened as Boyd has pointed out in Ophidia, and (c) the epithelioid cells which are present as irregular collections in the adventitia in Amphibia (Palme, '34) (and not definitely mentioned by Boyd in his paper) are aggregated into a well defined mass, fit to be called a carotid body, to which distinct nerve fibres are distributed, and in which blood sinusoids lie in between the rows of cells.

A comparison of figure 3, the carotid body of a cat, with figure 1 will help to appreciate how the carotid body of Varanus monitor approaches very nearly the mammalian organ in structure. In the cat, the carotid body is applied to the dorsal aspect of the internal carotid artery, its connective tissue stroma being directly continuous with the adventitia of the artery. The parenchymal cells are situated in groups around sinusoids. In Varanus monitor also we find the epithelioid cells arranged around blood sinusoids, and here the relation of the carotid body to the arterial wall is more intimate inasmuch as the connective tissue trabeculae are continuous with the media of the artery. Evidently the carotid sinus-carotid gland apparatus subserving pressoreceptive and chemoreceptive functions are very intimately blended together in the lower vertebrates and as we ascend higher (due to greater specialisation of function?) the two organs dissociate from one another.

The structure of the carotid gland and carotid sinus in Varanus monitor is distinctly a stage midway between that described by Boyd in Ophidia and the structure in mammals.

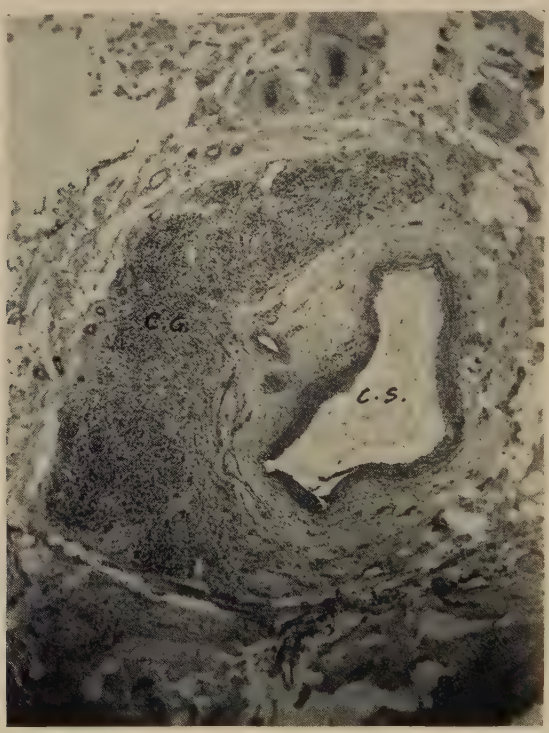

Fig. 3 Low power microphotograph of carotid gland of cat showing intimate relationship of the carotid gland (CG) and carotid sinus (CS).

SUMMARY

The carotid gland and carotid sinus of Varanus monitor is described with special reference to their structural affinities with the Ophidia on the one hand and the mammals on the other. The carotid sinus is present in the form of a dilatation of the lumen of the termination of the common carotid artery and commencement of the internal carotid artery, the walls of the sinus being uniform in outline. The carotid gland consists of a fusiform aggregation of epithelioid cells applied to

the dorsal aspect of the carotid sinus, and partially subdivided into separate loculi by fibromuscular trabeculae which radiate from the media of the artery into this mass. Within the group of cells are blood sinusoids. Nerve fibres are found to terminate as free nerve terminals both in the wall of the carotid sinus and in the fibromuscular trabeculae dividing up the epithelioid cell mass.

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ELECTROMYOGRAPHIC STUDIES OF M. BICEPS BRACHII DURING NORMAL VOLUNTARY MOVEMENT AT THE ELBOW

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TWO FIGURES

INTRODUCTION

The increased interest in muscles in recent years has served to focus attention on the paucity of good information on the behavior of muscles in the normal human individual during voluntary movements. A few individuals using different techniques have made contributions that are referred to frequently in the literature. The study of the response of individual muscles to electrical stimulation is perhaps highlighted by the work of Duchenne (1867) recently published in translation (Kaplan, '49). The analysis of muscles and joints on a purely mechanical basis was carried to a high level by Fick ('10, '11). The study by direct observation and palpation was very successful in the hands of Beevor ('03) and Wright ('28). There is a long and excellent literature on the properties of muscles removed from the body and in the experimental animal. Only in rather recent years has the study of muscles in the normal human individual through electromyography become at all common. While this offers many difficulties of interpretation it does give information that advances, but does not complete the picture of muscle function. An excellent discussion of the problems of the interpretation of the electromyogram has been given by Adrian ('25) and by Denny-Brown ('49), who, in addition, has contributed an adequate review of the literature.

Our purpose was to determine whether in a given movement a particular muscle was active and whether it was equally active throughout a movement, or whether it was more active during one phase of a movement. Appreciating that many variables enter into the picture when one uses surface electrodes it seemed important to know whether there was a "pattern" of potentials for a specific muscle under a given set of conditions, whether the pattern was much the same for different individuals and whether it was similar for the same individual at different times.

The term "pattern" is of necessity a loose one but there are in each record areas of higher and lower deflections variable in location and extent and perhaps the term covers the overall picture as well as any word can. It may be permissible to enumerate a few obvious things as a background for examining the records. Each muscle is made up of many units not all of which are necessarily active at the same time. In most instances there are several muscles capable of producing a given movement. In this situation no single muscle is necessarily active throughout the entire movement or if active throughout it need not be equally active in all phases. It must also be appreciated that there are factors of summation and cancellation and that chance must of necessity enter into the picture. There are undoubtedly subjective factors that that it is not possible to eliminate. Repeated experiments on the same individual does suggest however that there is a fair amount of regularity in relative amplitudes which can best be expressed by short verbal comments and the free use of illustrations.

SUBJECTS AND METHODS

Electromyograms were obtained from 8 normal subjects. All were students and faculty in the department of anatomy.

The electromyographs used were modified electrocardiographs. The basic unit was an early model electrocardiograph (Model A) manufactured by the General Electric Company. This photographic recording machine proved very satisfactory. Its viewing screen enabled the operator to determine if

conditions were satisfactory for a recording. The sensitivity of this unit was increased approximately 10-fold by the inclusion of an amplifier in the subject circuit. The modification made it necessary to work within a screened room. Interference was further reduced by a copper woven wire screen between the operator and the electromyograph. Most of the records were made at the speed customarily used for electrocardiographs (2.5 cm a second) but adjustment of the governor allowed film speeds up to 5.0 cm a second. A switch in the light circuit provided a means of interrupting the recording briefly at any point and thus was useful as a marker at the beginning and end of the movement. Three electromyographs were arranged so that they could be activated simultaneously.

Covered wire leads were used between the surface electrodes and the added amplifier. Electrodes were zinc disks 1.0 cm in diameter. These were held in place on the subject's skin by a liquid adhesive.

Action potentials from m. biceps brachii of the right side were led off through a pair of electrodes from each of the two heads. Markings for the placement of the electrodes were made with the biceps in full contraction. The vertical distance between the two electrodes forming the pair was 3.0 cm; the horizontal distance between the pairs was approximately 3.0 cm. The muscle belly was thus divided, for recording purposes, into a lateral portion continuous with the long head and a medial portion continuous with the short head. All records were taken with the subject in the sitting position. In the starting position for the flexion movements the arm was at the side in slight lateral rotation as in the anatomical position and in contact near the elbow with a bar which prevented extension of the arm beyond the plane of the trunk. The forearm was either in supination or in pronation. When the subject was relaxed in this position the muscle was essentially electrically negative. The starting position for the extension movements differed in that the elbow joint was flexed so that the angle between the arm and forearm was

about 45° and the biceps, instead of being electrically negative, showed moderate action potentials. When the operator was convinced, by observation of the galvanometer beam, that conditions were satisfactory for recording he signalled the subject to begin the movement. The subject signalled the operator at the end of the movement. The arm position for the holds was the same as for the movements; the elbow was in a position of partial flexion and the forearm in supination. There were of course potentials present as soon as the hold position was assumed.

Records were obtained during flexion (acceleration), hold (zero acceleration) and extension (negative acceleration) at the elbow. Flexion and extension were performed without additional load and with loads of 1.0 kg and 2.0 kg, with the forearm supine and with the forearm prone. The positions of partial flexion for the hold records were such that the angles between the arm and forearm were 135°, 90°, and 45° during three successive records. These were made without load and with a load of 1.0 kg.

RESULTS

Flexion — acceleration at the elbow

All of the electromyograms showed deflections which begin at or near the initiation of the movement and continue during the entire excursion of the forearm. The pattern of these deflections without added load (record 1) showed a gradual increase in amplitude during the first third of half of the record followed by an irregular plateau which was maintained to the end of the movement.

When a load of 1.0 or 2.0 kg was carried in the hand an overall increase in amplitude resulted (2, 3). The most conspicuous increase in amplitude occurred in the middle portion of the record when the movements were made with the forearm supine (2, 3).

The effect of the position of an accessory joint was demonstrated by the recording of a lower amplitude when the forearm was prone (4, 5, 6, 7).

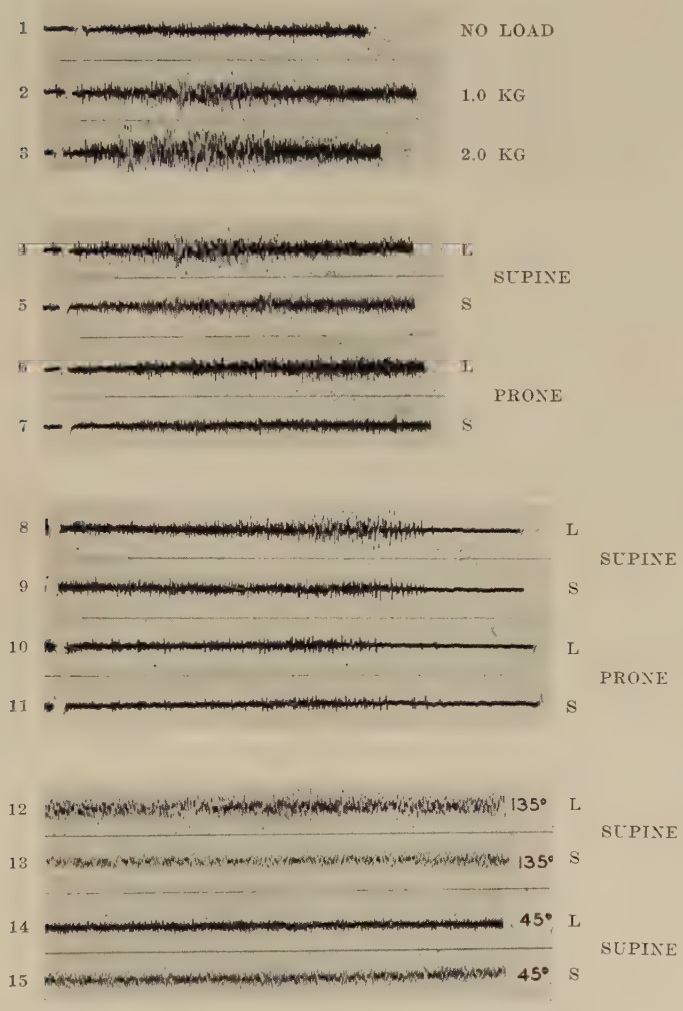

Fig. 1 Records from the same subject during one experiment.

1–3 Elbow flexion (acceleration), forearm supine, long head. Recording time for record 1 was 5.2 seconds. These records illustrate the effect of added load.

4–7 Elbow flexion (acceleration), added load 1.0 kg. These records illustrate the relative activity of the long head (L) and the short head (S) and the effect on the activity of the biceps when accessory joints (radioulnar), crossed by the muscle, are changed in position.

8–11 Elbow extension (deceleration) added load 1.0 kg. The general amplitude is less than in the acceleration series. The period of essentially zero potential is an interesting feature of the deceleration movement.

12–15 Hold (zero acceleration) added load 1.0 kg. These records illustrate the variable effect of the degree of flexion at the elbow on the activity of the biceps. The amplitude may be essentially the same as shown by the records of short head (13, 15) or different as shown by the records of the long head (12–14).

Usually the amplitude of the recording from the long head was slightly greater than that from the short head (4, 5), but the maximum amplitude for the series was recorded from the short head with the forearm supine and an added load of 2.0 kg.

When simultaneous recordings from the long and short heads were compared in detail the electromyograms of some movements were similar in pattern (6, 7). This similarity occurred more frequently when the forearm was in the prone position.

Extension — negative acceleration at the elbow

All of the electromyograms showed deflections of low to moderate amplitude preceding the movement because in the starting position the elbow joint was flexed with an angle of 45° between the arm and the forearm. The pattern of the electromyogram for this series was variable but the most frequent pattern can be divided into three areas. The first portion of the record shows a relatively low but fairly uniform amplitude; the second portion is a region of increased amplitude which builds up gradually and decreases more abruptly; the final portion is essentially at zero potential.

Of the records made without load only the recording from the long head with the forearm supine was characteristic of the above description. The rest of this series showed a relatively uniform, low amplitude over the first two-thirds of the record followed by zero potentials during the final third.

When added load was held in the hand the characteristic pattern occurred more frequently (8). Added load also produced a general increase in amplitude and a shortening of that portion showing zero potentials.

The effect of the position of the forearm was demonstrated by a lower amplitude when the forearm was prone (8, 9, 10, 11) and was most obvious in the 2.0 kg series.

The potentials recorded from the long head were greater than those from the short head during each movement in the deceleration series.

Hold — zero acceleration at the elbow

The electromyograms showed continuous activity whenever the muscle served to hold the forearm against the pull of gravity (12, 13, 14, 15). The individual deflections were irregular in amplitude, but usually were distributed in a manner which gave the total recording a relatively uniform appearance. Some of the records showed groups of 3–10 deflections of higher amplitude irregularly distributed.

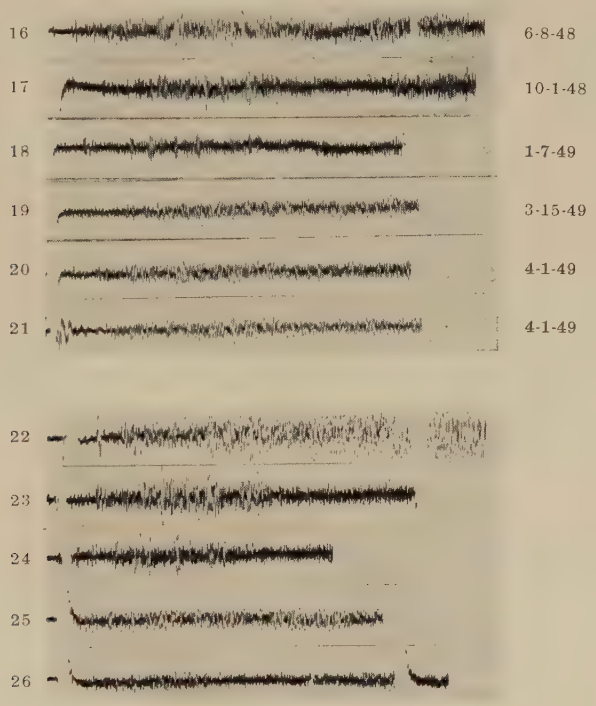

Fig. 2 Records of the same movement from a single subject at irregular intervals.

16–21 Flexion (acceleration) at elbow, forearm supine, short head, added load 1.0 kg. The patterns of activity shown in 16 and 17 differ from the others in having a secondary rise in potentials in the last part of the movement. The general amplitude is greater in the first two records than in the later ones. Total times for record 17: 7.4." The short interruption in 16 indicates the end of the movement. There is a brief recording of hold potentials after that.

22–26 Records of the same movement from different subjects.

Flexion (acceleration) at elbow, forearm supine, long head, added load 1.0 kg.

In many trials the activity was greatest in the 135° position (12, 14). In other trials the amplitude was essentially the same in all three positions.

Recordings from the same individual

Electromyograms were recorded under similar conditions and from the same subject at irregular intervals over a period of 10 months. These tracings showed variations in both amplitude and pattern (16–21). There was a progressive decrease in amplitude in each succeeding trial for the first three of the 6 trials. The last three trials were approximately equal in amplitude to the third. Records 16 and 17 are different in pattern from the others in the series in that each showed an area of higher deflections near the end.

Recordings from different individuals

Electromoyograms of the same movement recorded from different subjects varied in both amplitude and pattern. The degree of variation in amplitude may be as much as three-fold for equivalent areas of comparable records (22 and 26). A difference in pattern was obvious in some instances (22, 23) and not obvious in others (23, 24).

SUMMARY

Action potentials were recorded from m. biceps brachii of normal subjects during voluntary movements of the elbow joint. Repeated recordings from one subject at the same sitting were usually similar. Repeated recordings from one subject with intervals of weeks intervening usually, but not always, gave similar patterns. Action potentials tended to be higher on the earlier trials than on the later ones. When two or more snbjects were studied under similar conditions the recordings were similar or different in pattern and amplitude. The variations in pattern and amplitude were assumed to represent differences in the use of the muscle during a given movement. Potentials of lower amplitude were recorded from

both parts of the muscle when the movement was performed with the forearm in the prone position and, in general, from the short head under all conditions studied.

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EFFECTS OF THYROXIN INJECTIONS ON GROWTH AND DIFFERENTIATION OF THE SKELETON OF HYPOPHYSECTOMIZED FEMALE RATS [1]

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SEVEN FIGURES

The purpose of this experiment was to study the effects of thyroxin injections on skeletal growth and differentiation in hypophysectomized rats.

As noted in a previous paper (Ray, Simpson, Li, Asling and Evans, '50) the results of experiments conducted at the Institute of Experimental Biology (Evans, Simpson and Pencharz, '39; Becks, Ray, Simpson and Evans, '42) and those conducted by Laqueur, Dingemanse and Freud ('41) have been at variance. The Amsterdam group have reported that they were able to obtain growth in hypophysectomized rats with "thyranon pro injectione" (N. V. Organon in Oss), a proteolytic degradation product of thyroid gland. This substance was injected at a daily dose of 2.5 to 5 μg of organic bound iodine. The exact thyroxin equivalent of this dosage cannot be computed. The increase of weight of 8 hypophysectomized male rats during three weeks of injections averaged 16 gm. Repeated experiments by the Berkeley group, on the other hand, have failed to confirm this finding. The essential difference between the experiments conducted by the two groups may have been the dose level of thyroid hor-

[1] Aided by grants from the United States Public Health Service Grant RG-409 (C2) and the Research Board of the University of California.

mone injected — that used at Berkeley being from 5 to 7 μg of thyroxin per day.[2] Several recent experiments (Scow et al., '49; Ray et al., '50) have shown that the optimum dose of thyroxin in thyroparathyroidectomized rats was from 2.5 to 3.0 μg a day. Accordingly in the present experiment it was decided to use the same dose in hypophysectomized rats to determine if the effects were different from those observed at the higher dose levels.

METHOD

Ten female rats of the Long-Evans strain were hypophysectomized at 21 days of age. Five of the animals were injected daily with thyroxin. The other five served as uninjected controls. A third group of three unoperated uninjected animals of the same chronological age was included as normal controls. During the experimental period, each group of animals was kept in a single heated cage, and all were fed the same diet. During the last 17 hours of the experimental period, the total food consumption of the animals for each of the three groups was determined.

Crystalline thyroxin (Squibb) was used, dissolved in a slightly alkaline aqueous solution. Three micrograms were injected subcutaneously daily. Injections were started at a chronological age of 30 days and continued for 30 days.

The animals were weighed every 5 days. At 30, 46, and 60 days of age each animal was anesthetized and roentgenograms taken. From the films the skeletal age and the length of the various parts of the skeletons were determined. At 61 days of age, the animals were autopsied. The tibia, humerus, third metacarpal and third costochondral junction were removed for histological study and, in addition, the right femur was preserved for chemical analysis. The technique of prepara-

[2] In a recent study (Asling, Becks, Simpson, and Evans, '49) prolongation of the injection period to 71 days at the 5 μg/day dose level resulted in body lengths of the injected animals which exceeded those of the hypophysectomized controls by a slight, statistically significant amount. Body weights remained the same as in the controls.

tion of the histological sections and method of gravimetric analysis for water, fat, organic content and ash have been described in a previous report (Ray et al., '50).

RESULTS

During the experimental period the animals receiving thyroxin were more active and had better appetities than the uninjected hypophysectomized rats. The total food intake for the injected group during the last 17 hours of the experimental period was 6.33 gm/rat as compared with 4.33 gm/rat for the uninjected group.

The average skeletal age, body weight and body length determinations are given in table 1.

The thyroxin-injected rats not only ate more but also gained more weight than the uninjected animals, the mean weight gain during the experimental period being 20 gm for the injected rats as opposed to 9 gm for the hypophysectomized controls. As can be seen from figure 1, the gain in weight of the injected group occurred during the last 20 days of the experimental period.[3]

The thyroxin-injected rats also significantly exceeded their uninjected controls in body length (table 1).

In addition to the increase in length of the axial skeleton, measurements from the roentgenograms of representative bones of the appendicular skeleton revealed an increase in length following thyroxin injections in the hypophysectomized rats. The figures for the ulna, tibia, and the 6th caudal vertebra are given in table 2.

It is instructive to compare the growth of these two groups of rats with the skeletal maturation which they attained. As shown in table 1, the skeletal age of the uninjected hypophysectomized rats was 42 days at 60 days of chronologic age.

[3] The best growth only approached the unit of growth (1 gm per day) during the last part of the experimental period. Even here it was only demonstrated by using a dose lower than those previously employed, whereas it is a characteristic of the hypophyseal growth hormone that increasing doses give proportionate increases in body weight.

TABLE 1

Estimated skeletal age, and average body weight and length of hypophysectomized female rats injected with thyroxin [1]

GROUP	NO. OF RATS	SKELETAL AGE		BODY WEIGHT		BODY LENGTH AT AUTOPSY		
		At 30 days	At 60 days	At autopsy	Gain during injection period	Nose to anus	Anus to tail-tip	Total
		days	*days*	*gm*	*gm*	*mm*	*mm*	*mm*
Hypophysectomized, uninjected	5	29 ± 0.9 [2]	42 ± 1.3	65 ± 2.4	9 ± 1.2	134 ± 2.0	112 ± 2.8	246 ± 4.0
Hypophysectomized, thyroxin, 3.0 μg per day	5	29 ± 1.4	55 ± 2.2	78 ± 2.5	20 ± 1.4	147 ± 1.3	125 ± 3.1	272 ± 3.9
Intact, uninjected	3	30 ± 0.0	60 ± 0.0	152 ± 11.7	85 ± 10.0	185 ± 5.1	164 ± 5.9	349 ± 10.8

[1] Hypophysectomized when 21 days of age; injected subcutaneously with thyroxin from 30 to 60 days of age.
[2] Average of 4 rats.

TABLE 2

Average lengths of ulna, tibia, and 6th caudal vertebra of hypophysectomized female rats injected with thyroxin [1]

GROUP	NO. OF RATS	ULNA			TIBIA			6TH CAUDAL VERTEBRA		
		At 30 days	At 46 days	At 60 days	At 30 days	At 46 days	At 60 days	At 30 days	At 46 days	At 60 days
		mm	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*
Hypophysectomized, uninjected	5	20.9 [2] ± 0.17	21.4 ± 0.25	21.7 ± 0.26	24.4 [2] ± 0.26	25.3 ± 0.24	25.9 ± 0.30	5.2 [2] ± 0.10	5.5 ± 0.13	5.7 ± 0.08
Hypophysectomized, thyroxin, 3.0 μg per day	5	21.1 ± 0.10	22.0 ± 0.14	22.8 ± 0.08	24.6 ± 0.21	26.8 ± 0.22	28.0 ± 0.22	5.2 ± 0.13	6.0 ± 0.08	6.3 ± 0.16
Intact, uninjected	3			26.9 ± 0.53			32.4 ± 0.54			8.6 ± 0.12

[1] Hypophysectomized when 21 days of age; measurements made on roentgenograms taken at intervals during the injection of thyroxin from 30 to 60 days of age.
[2] Average of 4 rats.

Their size (body weight, length, and tibia length) resembled that of much younger rats, approximately 30 days of age. This indicated that growth was depressed by hypophysectomy to a greater extent than was skeletal maturation. The thyroxin-injected rats had skeletal ages of 55 days at 60 days chronological age, though their bodily dimensions were like those of 33-day old normal rats. This indicated that the thyroxin stimulated skeletal maturation more than it stimulated growth.

Since the main basis for the roentgenographic criteria of skeletal age was the degree of development of the secondary

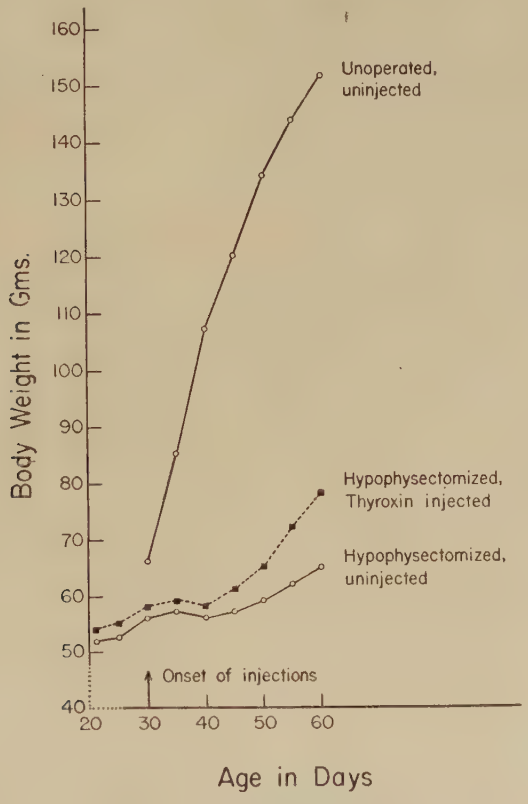


Fig. 1 Curve showing the average growth of 5 hypophysectomized rats injected with thyroxin compared with that of 5 uninjected hypophysectomized and three normal rats during the 30-day experimental period.

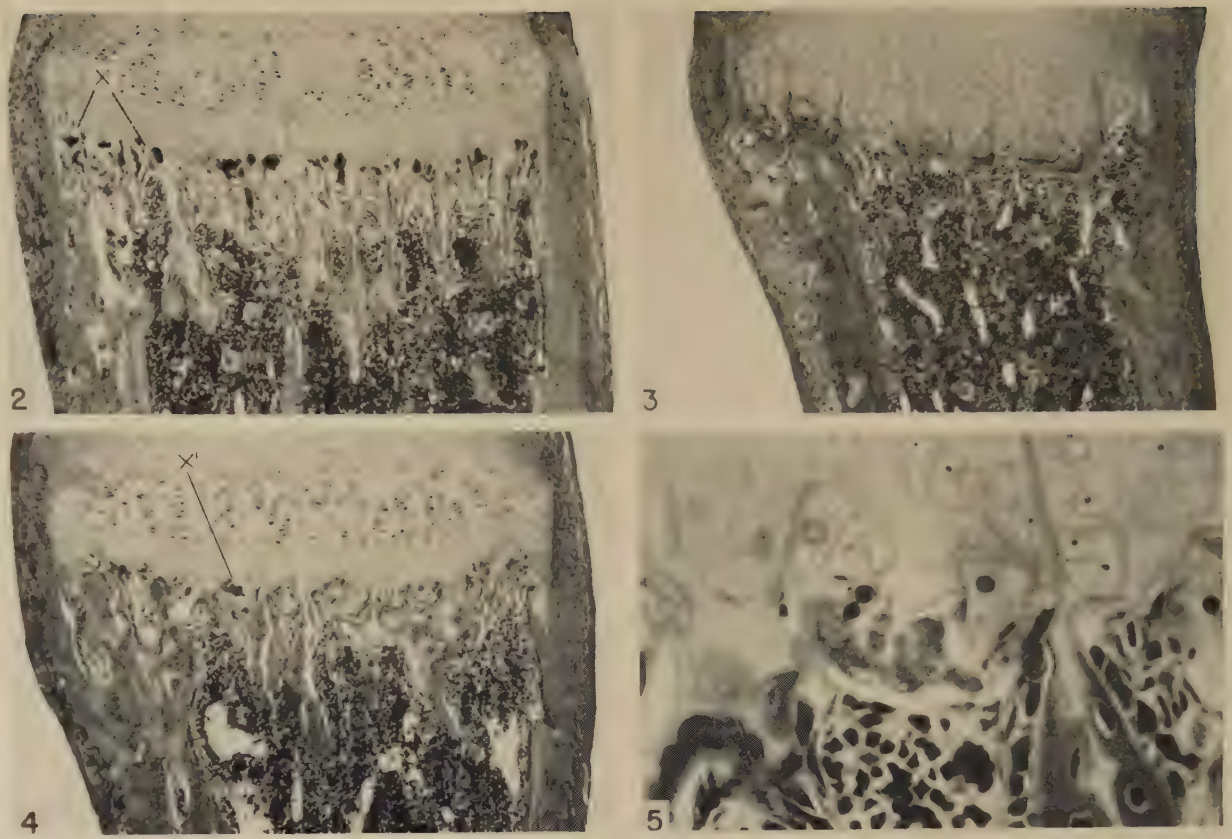
(epiphyseal) ossification centers, the histogenetic means by which this advance in skeletal differentiation occurred was of special interest. Several regions of endochondral ossification (proximal end of tibia, third metacarpal, distal end of humerus, and costochondral junction) were therefore sectioned for histological study.

In the tibia, the main interest was in the width and activity of its proximal epiphyseal plate. If differentiation were progressing faster than proliferation, the plate should be narrower than in the controls (i.e., cartilage removal should be more rapid than cartilage formation). Such a narrowing was actually observed, but in this experiment it did not become statistically significant.[4] The width in the thyroxin-injected rats was $258 \pm 0.73\ \mu$, compared with $273 \pm 22.5\ \mu$ in the hypophysectomized controls, and $275 \pm 28.9\ \mu$ in the normal rats. Histologically the thyroxin-injected rats showed more marrow in contact at the erosion zone of the cartilage plate than did the hypophysectomized controls, but did not approach the normal rats in this respect.

Premature epiphyseal closure was not found at the metacarpal epiphysis. The roentgenographic estimate of the skeletal age (55 days) at 60 days of chronological age did not justify one in expecting closure, since the normal time of closure is 90–110 days (Becks, Asling, Collins, Simpson and Evans, '48).

Histologic support of the roentgenographic indications of skeletal aging was gained from study of the distal end of the humerus. In the hypophysectomized controls fragments of the main epiphyseal plate still remained at 60 days of age, although normally these are all removed by 40 days of age (Becks, Asling, Simpson, Evans and Li, '48). In the thyroxin-injected rats all such fragments had been removed. In one animal of this group, premature closure of the medial

[4] In a study to be reported with C. P. Williams, thyroxin injections starting immediately after hypophysectomy and continuing for 40 days did result in a significant reduction of the cartilage plate.



Figs. 2–5 Third left costochondral junction of 60-day old female rats. Frontal sections, Mallory stain, × 80, (except as otherwise stated.
Fig. 2 Normal. (Sp. 813)
Fig. 3 Hypophysectomized at 21 days of age, no injections. (Sp. 808)
Fig. 4 Hypophysectomized at 21 days of age, injected with 3 μg of thyroxin daily from 30 to 60 days of age. (Sp. 803)
Fig. 5 Higher magnification (× 475) of multinucleated chondroclast shown at X′ in figure 4.

epicondylar epiphysis of the humerus was found; normally this cartilage remains intact until 102–135 days of age.

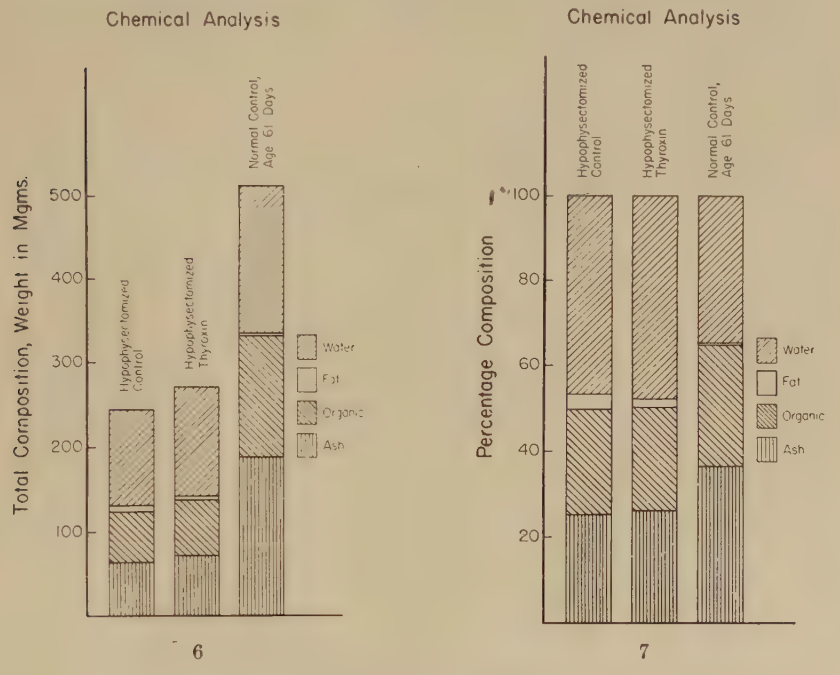
The most marked evidence of excessive cartilage erosion was observed at the costochondral junction (figs. 2–5). Here the hypophysectomized controls showed complete cessation of endochondral ossification, the cartilage margin being sealed from the marrow cavity by bone. The injections of thyroxin produced slightly greater proliferation of cartilage, and also prevented its sealing-off by bone. Marrow contact and cartilage erosion was maintained. The marrow tufts were more cellular than vascular.

Figures 2 to 5 illustrate the costochondral junction in a normal rat, a hypophysectomized control, and a thyroxin-injected hypophysectomized rat. Figure 2 shows a balance between cartilage formation, cartilage erosion, and osteogenesis characteristic of normal endochondral bone growth. At the top of the picture small nests of reserve chondrocytes are seen. Next, in the zone of proliferating cells the flattened chondrocytes are found disposed in rows and conical clusters. Finally there may be seen two or three layers of enlarged, rounded chondrocytes with a vacuolated cytoplasm and faint-staining nuclei. Tufts of marrow are in contact with these cells along a regular margin, the erosion line. Blood-filled capillaries are seen in most of these tufts. Although not shown in detail at this magnification the tufts often contain large, irregularly shaped, multinucleated cells. Two such are indicated by the lines marked X. Deeper in the marrow space the elongated bony trabeculae of the spongiosa are seen, at first straight and delicate but subsequently anastomosing. In the primary spongiosa most of the trabeculae contain cores of cartilage matrix, continuous with the longitudinal partitions of the cartilage in the zone of enlarged cells.

In figure 3 the cartilage in the untreated hypophysectomized rat shows poor alignment of cells. A layer of bone seals the cartilage from the marrow cavity. No bony trabeculae are seen.

Figure 4 illustrates the response to injection of thyroxin. The cartilage is more like that in normal rats. Abundant marrow is in contact with the cartilage. Osteogenesis is active, although the arrangement of trabeculae is not as regular as in normal rats. An important feature shown in these rats is the presence of many chrondroclasts at the line of cartilage erosion. One such cell is seen at X′, and is shown in these rats in higher magnification in figure 5. These cells are multinucleated, have a vacuolated cytoplasm, and are ir-

regularly shaped. Often the vertical striations of the "brush border" may be seen against the matrix. They resemble osteoclasts in all respects save their contact with cartilage instead of bone. They were seen more clearly in the injected hypophysectomized rats than in normal rats; in the latter, although present in the marrow tufts, they were often obscured by the capillaries accompanying them. In the uninjected hypophysectomized rats they were scarce.



Figs. 6 and 7 Chemical composition of the femur of hypophysectomized rats uninjected and injected with thyroxin, and of normal rats of the same age (60 days.

Fig. 6 Composition by weight.

Fig. 7 Percentage composition.

Chemical analysis of the femur did not demonstrate any difference in the bones of thyroxin-injected and untreated hypophysectomized rats. Their water, fat, remaining organic material, and ash contents are shown by graphs in figure 6 (average weight, in milligrams) and figure 7 (percentage composition). Both groups showed relatively greater water

content, and less organic material and ash than in the group of normal animals. The calcium and phosphorus contents of the ash were similar in all three groups of rats.[5] Expressed in milligrams per 100 mg of ash, the calcium values were 40.2 ± 0.75, 39.7 ± 1.04, and 37.8 ± 0.10 for the uninjected hypophysectomized, the thyroxin-injected hypophysectomized, and the normal rats, respectively. The corresponding values for phosphorus were 16.6 ± 0.84, 17.2 ± 0.12, and 16.7 ± 0.20. respectively.

SUMMARY

A study of the effect of thyroxin in producing skeletal growth and differentiation in hypophysectomized female rats was conducted by comparing body dimensions and roentgenographic, histologic, and chemical analyses of the skeletons with those of untreated hypophysectomized and normal rats.

The untreated rats (hypophysectomized at 21 days of age and sacrificed at 60 days of age) showed marked retardation of growth in body weight and length, and a delay, though less pronounced, in skeletal maturation (judged roentgenographically). The hypophysectomized rats treated with thyroxin (3.0 μg/day between 30 and 60 days of age) showed an increase in appetite and a slight gain in body weight and body length over the hypophysectomized untreated rats. On the other hand, there was a marked gain in skeletal maturation.

Histologic studies of the proximal end of the tibia, third metacarpal, distal end of humerus, and costochondral junction showed arrest of endochondral ossification in the uninjected hypophysectomized rats. In the thyroxin-injected rats, erosion of cartilage and replacement by bone continued, though chondrogenesis was not maintained at an equal rate. This accounted for the greater differentiation of their skeletons demonstrated roentgenographically.

[5] We acknowledge gratefully the assistance of Dr. Harold D. Copp, Division of Physiology, University of California, in the analyses of calcium and phosphorus in the bone-ash samples.

On chemical analysis of the femur, both uninjected and injected hypophysectomized rats had more water and less organic matter and ash than normal. No evidence of chemical maturation of the skeleton was therefore found.

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A MORPHOLOGICAL AND CYTOCHEMICAL STUDY OF THE POSTNATAL DEVELOPMENT OF THE RAT'S ADRENAL CORTEX [1]

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SIXTEEN FIGURES

This paper reports the results of a histological and cytochemical study of the adrenal cortex in the albino rat during development from birth to 6 weeks of age. The observations refer primarily to the question of the zonation of secretory activity in the cortex, as affected by the growth and reorganization of the gland after birth. Zonation of the cortex is of particular interest because studies on the adult rat have suggested that while both the zona glomerulosa and zona fasciculata secrete steroid hormones, they serve different functions and are separately controlled (Deane, Shaw and Greep, '48; Greep and Deane, '49a). Earlier studies on the cortex of the young rat (Jackson, '19; Engström, '36; Tobin and Whitehead, '42; and Mitchell, '48) indicate that zonation of the cortex, with respect to both histological organization and lipid content, is present from birth. These investigators were not concerned with the functional separation of the two zones, however, nor did they attempt to judge whether the cortex is actively secreting in infancy. We have therefore used cytochemical techniques designed to reveal hormone precursors (Deane, Shaw and Greep). A morphological study of these adrenals was made also so that our series of animals could be compared with those of the earlier reports.

[1] This work was done in part under a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

In addition, because of the difference of opinion concerning the occurrence of a fetal cortex or X zone in the rat adrenal gland (Howard, '38; Tobin and Whitehead), attention has been paid to any signs which might distinguish the inner part of the cortex from the outer zones.

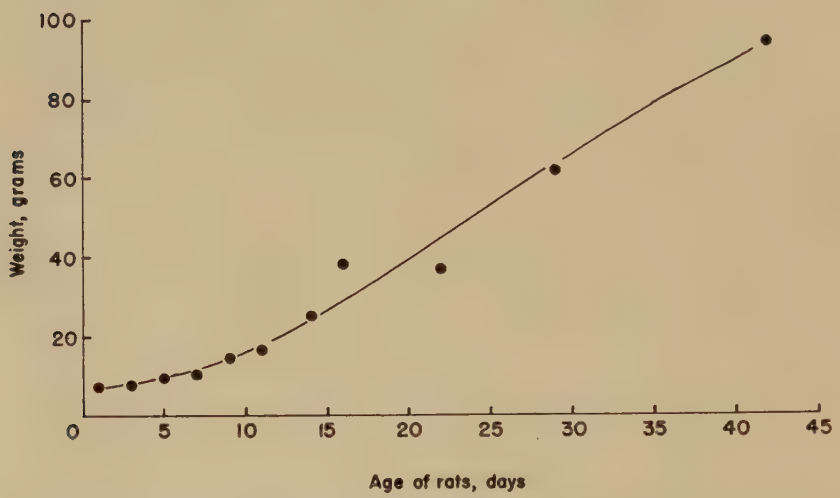

Fig. 1 Each dot represents the average weight of the 4 young rats at the time they were killed. The approximate growth curve of the animals has been drawn in.

MATERIAL AND METHODS

Rats of the Sprague-Dawley strain that were fed Purina laboratory chow were used. Following breeding, female rats were placed in separate cages. The number of young in each litter was reduced to 6 immediately after birth. The mother was left with her young for 30 days.

Four young rats were killed at each of the following ages: 1, 3, 5, 7, 9, 11, 14, 16, 22, 29 and 42 days. For each age group, two animals were taken from one litter, two from another — in each instance one male and one female. The average weights of the rats used are presented in figure 1.

The rats were killed by decapitation. One adrenal gland from each was fixed overnight in Bouin's fluid, dehydrated,

embedded in paraffin, and sectioned serially at 10 μ. The sections were stained with Harris' hematoxylin and eosin.

The other adrenal was fixed in 10% neutralized formalin for a week and then sectioned, without any embedding medium, on the freezing microtome at 15 μ. Sections including the medulla were prepared in the following ways: (1) stained for one minute with a 1% solution of sudan black B in 70% alcohol, (2) stained by the Schultz cholesterol method, (3) stained by the Ashbel-Seligman ('49) method for ketonic steroids, (4) mounted unstained, and (5) extracted for one-half hour with acetone at room temperature and then mounted. The unstained sections were compared with those that had been extracted with acetone under the polarizing and fluorescence microscopes. These methods as a group are believed to localize the sites of formation of the cortical hormones; they have been found to aid in evalulating the secretory activity of the gland as well (Deane, Shaw and Greep).

Mitotic counts were made on the hematoxylin and eosin preparations of adrenal glands from two rats of each age group. Four sections near the middle of each gland were studied under the oil-immersion objective. All unquestionable mitotic figures in metaphase and anaphase were recorded as to their position in the cortex, i.e., in the zona glomerulosa (including the transitional zone) or in the zona fasciculata. The area of the cortex in these same sections was measured with a planimeter on a × 40 outline made with the aid of the camera lucida. The mitotic ratio was then calculated as the number of mitotic figures per square millimeter of cortex.

RESULTS

No differences, either histological or cytochemical, were detected between the adrenal glands of the male and female rats of the same age. Consequently the descriptions which follow are based on study of the glands from all 4 animals of each age group.

Morphology of the developing adrenal cortex

At birth, the rat's adrenal gland is bounded by a well-defined capsule and contains both cortical and medullary tissue. The medulla is still incompletely organized, however, with the center of the gland consisting of strands of cortical cells intermixed with clumps of medullary cells. This intermixture is best seen in frozen sections stained with a sudan dye, by reason of the fatty inclusions in the cortical cells (fig. 2). During the first week after birth the medulla becomes unified, while the cortical cells located in the central area shrink and gradually disappear (fig. 3). After the first week, cortical cells are only occasionally seen within the medulla (figs. 4 and 5).

From birth, the cortex is separable into two zones, a narrow outer glomerulosa and a broad inner fasciculata. Initially the cells of the glomerulosa are small and possess few vacuoles. They are crowded together without apparent organization (fig. 6). Before the end of a week they acquire numerous fat droplets (fig. 3) and enlarge (fig. 7). Gradually they are organized into the small balls and arcs which characterize the zone in the adult cortex (figs. 8 and 9).

When the cells of the glomerulosa become fatty, a few rows of small spindle-shaped cells which are devoid of fat persist between the glomerulosa and the fasciculata (figs. 3 to 5 and 7 to 9). This zone, which we shall call the transitional zone (Greep and Deane, '49a), was termed the Grenzschicht by Engström and the zone of compression by Mitchell.

The fasciculata is composed initially of uniformly large, vacuolated cells which lack apparent organization (fig. 6). Within a week, distinct radially-directed cords develop (fig. 7). They follow a straight course to the border of the medulla (fig. 10) until the end of the second week, when a small zona reticularis develops (fig. 11). This reticulate organization of the cords at the border of the medulla becomes more prominent in the older animals (fig. 12). At no time, however, are the staining properties of this zone different from those of

the rest of the cortical cells in hematoxylin and eosin preparations. Moreover, the nuclei in this region resemble those lying more peripherally in the cortex (fig. 8). Even at the end of 6 weeks, the signs of cellular degeneration which often characterize the reticularis in the adult animal have not made their appearance.

TABLE 1

The number of mitotic figures in the capsule and in the cortex of the adrenal gland, the cortical area and the mitotic ratio in sections of adrenals from young rats. Four adrenal sections from each of two rats per age group were studied and the results averaged

AGE OF RATS IN DAYS	NUMBER OF MITOSES PER SECTION				AREA OF CORTEX PER SECTION IN MM^2	MITOTIC RATIO: NUMBER MITOSES PER MM^2 CORTEX
	Capsule	Adrenal cortex				
		Glom.[1]	Fasc.[1]	Total		
1	2.0	6.5	1.7	8.2	0.554	14.8
3	4.0	7.0	4.4	11.4	0.578	19.7
5	2.8	4.0	1.0	5.0	0.512	9.8
7	2.2	5.5	8.9	14.4	0.544	26.5
9	2.0	7.8	5.0	12.8	0.616	20.8
11	0.8	6.8	6.6	13.4	0.963	13.9
14	1.5	15.0	25.6	40.6	1.125	35.0
16	2.6	10.1	17.9	28.0	1.548	18.1
22	0.7	9.9	14.8	24.7	2.213	11.1
29	0.4	13.9	4.8	18.7	3.047	6.1
42	0.2	0.5	1.5	2.0	3.245	0.6

[1] Abbreviations: Glom. = zona glomerulosa (plus the transitional zone). Fasc. = zona fasciculata.

Mitotic figures are frequently observed in the glands of rats up to three to 4 weeks of age, but have almost entirely disappeared in the animals 6 weeks old. They occur both in the capsular fibroblasts and in the cortical parenchyma (figs. 6 to 9). In the cortex, divisions are most common in the glomerulosa and outer fasciculata. They occasionally appear at deeper levels, however, especially in animals two weeks of age and older (fig. 8). They have also been observed, though infrequently, within the transitional zone.

The average number of mitotic figures (metaphases and anaphases) occurring in the capsule and in the two principal

zones of the cortex are given in table 1. In the capsule, they are relatively infrequent in the younger rats and virtually disappear after three weeks. In both the glomerulosa and fasciculata cell divisions are numerous in rats up to 4 weeks of age. When the mitotic ratio is calculated as the number of mitoses in the cortex (exclusive of capsule) to the cross-sectional area, peaks in mitotic activity are seen to occur at three days, at 7 days, and again at 14 days. The latter two increments are associated with periods of marked enlargement of the gland, as revealed by the cross-sectional areas listed in the table and also by comparison of the figures 3 to 5 and 10 to 12. Cell multiplication during the first week appears to be offset by the degeneration of cortical cells lying in and around the medulla (compare figs. 2 and 3).

Lipids in the developing adrenal cortex

At the time of birth, the cells of the zona glomerulosa contain only occasional small fat droplets, whereas those of the rest of the cortex are uniformly crowded with small lipid droplets (fig. 2). By 5 days, the glomerulosa has acquired a considerable amount of lipid (fig. 3) and remains fatty thereafter (figs. 4 and 5). After about two weeks, the zona fasciculata develops a gradient of fat content, with more persisting in the outer portion than further inward.

From birth, the lipid droplets in both zones display additional reactions which are characteristic of steroid-producing cells, i.e., the Schultz reaction for cholesterol and related substances (fig. 13), the Ashbel-Seligman reaction for ketonic steroids (fig. 14) and birefringence (figs. 15 and 16). In the youngest animals, the staining reactions are faint and the birefringent crystals exceedingly fine. With progressing age the staining becomes more intense and the crystals coarser. Only in the 6-week-old rats, however, has it been possible to detect acetone-soluble fluorescent materials.

As the medullary tissue coalesces during the first week of life, the cortical cells lying within the medulla and those lo-

cated on its border become more intensely fatty than those lying in the fasciculata proper (fig. 3). These intensely fatty cells gradually diminish in number and after the second week occur only in isolated groups (fig. 4). The droplets in these cells are larger than those found in the rest of the cortex. Initially these droplets give the Schultz test, stain bluish with the Ashbel-Seligman reagents and contain birefringent crystals. By the end of two weeks, however, even though large fat droplets remain in some cells, these "steroid" reactions are negative (figs. 13 to 15).

By 6 weeks lipid droplets have appeared in the cells of the zona reticularis in some of the rats (fig. 5). These droplets are small and display the special reactions (fig. 16).

DISCUSSION

In the adrenal cortex of the rat the zona glomerulosa is distinguishable from the zona fasciculata from the time of birth by reason of its morphological appearance and lipid content. Initially, the outer zone contains little lipid, in contrast to the fasciculata. Within the first week the glomerulosa acquires lipid and becomes separated from the fasciculata by a transitional zone of small, fat-free cells. The description of our material corresponds essentially to that given by Jackson, Engström, and Tobin and Whitehead.

The cytochemical tests we have used demonstrate that, at all ages, the fatty droplets in both the glomerulosa and in the fasciculata display reactions which are characteristic of ketosteroids. Not only do they give the Schultz cholesterol test, as reported by Tobin and Whitehead and Harrison and Cain ('47), but they react with a carbonyl reagent and also contain birefringent crystals. Fluorescence is not detectable until the rat is 6 weeks of age; this reaction, however, is relatively insensitive. At first the droplets are small, the special staining reactions weak and the birefringent crystals fine; gradually the droplets enlarge, stain more darkly and contain coarser crystals. Droplet size and coarseness of the birefringent crystals have been found to be correlated with

secretory rate: small droplets characterize rapid secretion, larger ones a slower release (Deane, Shaw and Greep). The presence of only small droplets of reactive lipid in the cortex at the time of birth suggests that secretion of cortical hormones is relatively rapid at this time. Later, as the droplets and the birefringent particles enlarge, the rate of turnover may be somewhat slower. The emergence of fluorescence at 6 weeks also suggests that some storage is occurring by this age. While we have no physiological evidence for these conclusions, tests on the urine of human infants reveal the presence of glycogenic steroids of cortical origin on the first day after birth (Venning et al., '49). Moreover, Jailer ('49) has shown that pituitary adrenocorticotropic hormone stimulates the adrenal in the 4-day-old rat. Adrenocorticotropin causes the release of glycogenic hormones in the rat, and these steroids appear to be formed by the zona fasciculata (Bergner and Deane, '48). No information is available, so far as we know, on the secretion of salt-regulating hormones by the adrenal cortex of the infant. These hormones are believed to come from the glomerulosa, and their release does not depend on the pituitary (Deane, Shaw and Greep).

No signs of an X zone have been detected in these young rats. The cells which initially lie within the medulla and on its border become increasingly fatty and gradually disappear. The lipid dropets in these cells, however, at first display special reactions suggesting the presence of steroids. The fat which makes its appearance in the X zone of the mouse as it undergoes involution displays none of these reactions (Jones, '49). Moreover, we have been unable to discern any distinctive staining properties or nuclear differences in the juxtamedullary cells which would suggest the existence of a peculiar zone such as that described by Mitchell. We therefore find ourselves in agreement with Tobin and Whitehead that the rat's cortex does not develop an X zone, as was claimed by Howard. We are unable to assess Jackson's suggestion that it is pressure exerted by the growing medulla

which causes the degeneration of the innermost cells of the cortex immediately after birth.

During the first week of life the cross-sectional area of the adrenal cortex does not appear to enlarge, although cell division is moderately frequent. Apparently a balance exists between cell multiplicatilon on the one hand and cell degeneration at the medullary border on the other. During the next three weeks, however, the cortex grows rapidly (table 1). Jackson found that the cortical cells do not enlarge significantly after birth, and our preparations confirm this (plate 2). Mitotic division in cortical cells, however, is frequent during this period, and the mitotic ratio reaches its highest peak at approximately 14 days. The divisions are distributed throughout the cortex. although, as in older rats (Baxter, '46; Mitchell), the majority lie in the glomerulosa and outer fasciculata. Both Engström and Mitchell describe mitosis as absent in the transitional zone, although present both outside and below this region. While we have occasionally seen mitotic figures in the transitional zone, our observations in general confirm theirs. These results lend support to the hypothesis that the normal growth of the cortex involves cell division within the respective zones, not a migration from the glomerulosa inward (Greep and Deane, '49a, b). Moreover, since cell division is relatively rare in the capsule, it may account solely for the growth of that structure as the cortex enlarges, rather than serving as a source of new parenchymal cells.

SUMMARY AND CONCLUSIONS

The adrenal cortex of young male and female rats aged one to 42 days has been studied histologically and cytochemically. No sex differences have been detected in the glands.

From birth the cortex is separable into two major zones, a glomerulosa and a fasciculata. The glomerulosa contains little lipid initially but acquires it during the first week. The fasciculata is uniformly fatty at birth; later a descending gradient develops, with more fat persisting in the outer portion than further inward. The cortical cells lying in and

around the medulla at the time of birth degenerate during the first two weeks of life. Neither these cells nor those which thereafter lie at the medullary border have the appearance of an X zone, contrary to the conclusion of Howard.

The lipid droplets in both the glomerulosa and fasciculata display reactions which are characteristic of ketosteroids. The droplet size is initially small, suggesting that secretion is rapid. Later the droplets enlarge, indicating some storage.

The cortex grows rapidly between 7 and 28 days. The growth depends principally on cell division. Mitotic figures occur in both the glomerulosa and fasciculata, suggesting the absence of cell migration. Mitosis is relatively rare in the capsule.

It is concluded that the functional zonation which characterizes the cortex of the adult rat extends back to birth and that the cells in both the fasciculata and glomerulosa are actively secreting in the infant adrenal.

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PLATE 1

EXPLANATION OF FIGURES

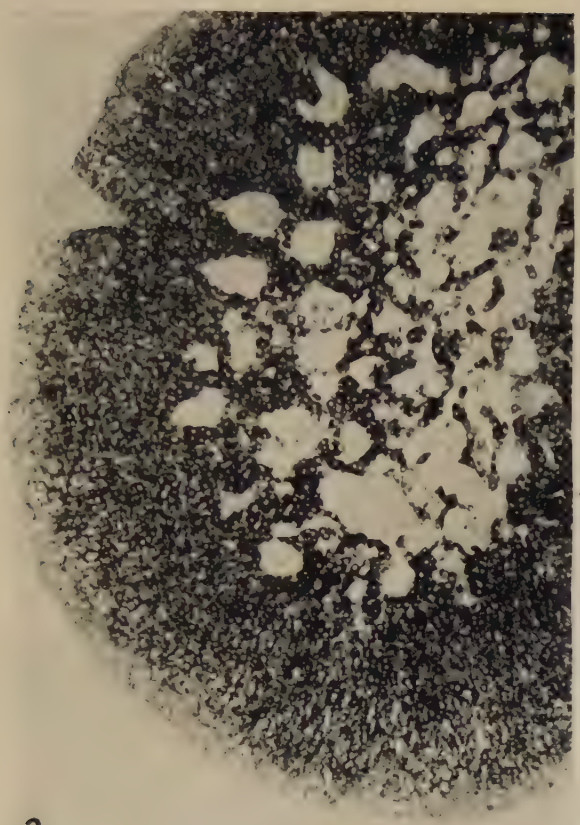
All figures on this plate are from adrenal glands that were fixed in 10% formalin, sectioned at 15 μ on the freezing microtome, and stained with sudan black B. Photographed with a blue filter (Wratten H). $\times$ 95.

2 Female rat, one day old. The adrenal gland is composed of fatty cortical cells and fat-free medullary cells. In the center of the gland, cortical strands are intermixed with the medullary tissue. The outermost zone of the cortex, the glomerulosa, contains relatively little lipid; the rest of the cortex is uniformly fatty.

3 Female rat, 5 days old. The medulla is now well defined; only a few cortical cells remain within it. These cells and those immediately adjacent to the medulla are more intensely fatty than the other cortical cells. The glomerulosa contains considerable amounts of lipid and is separated from the fasciculata by a relatively fat-free transitional zone.

4 Female rat, 16 days old. The cortex presents the appearance characterizing the adult gland, with a well-defined glomerulosa, transitional zone and fasciculata. The outer part of the fasciculata contains more lipid than the inner part. A few intensely fatty cells remain at the border of the medulla and within it.

5 Male rat, 42 days old. The cortex has enlarged but otherwise resembles that shown in figure 4. The inner fasciculata contains little fat, while the cells of the reticularis display small amounts.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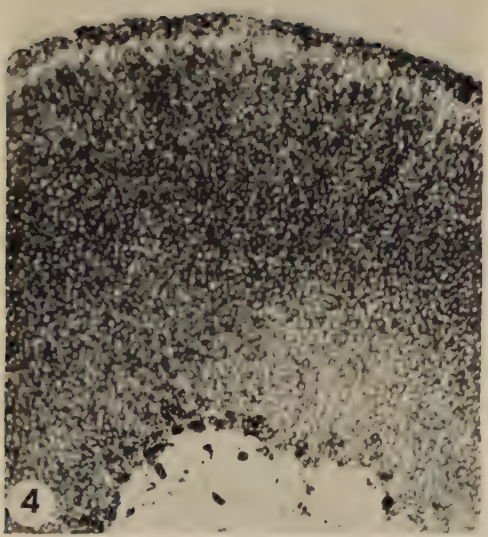
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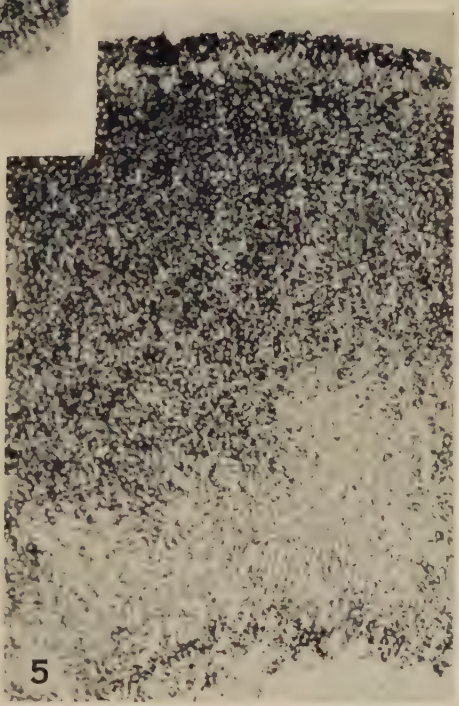
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PLATE 2

EXPLANATION OF FIGURES

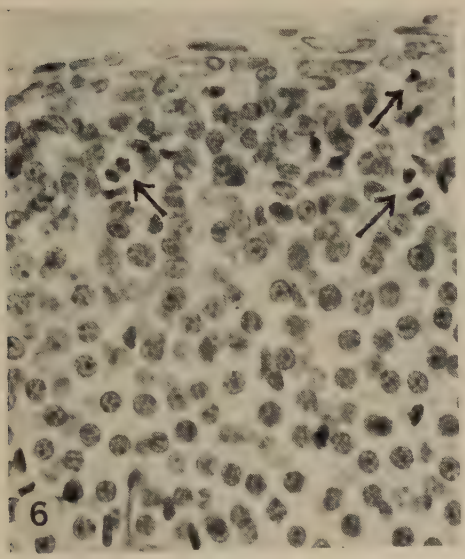
All figures on this plate are from adrenal glands that were fixed in Bouin's fluid, sectioned in paraffin at 10 μ and stained with Harris' hematoxylin and eosin. Photographed with an orange filter (Wratten E). $\times$ 480.

6 Female rat, three days old. The capsule, glomerulosa and outer fasciculata are shown. The cells of the glomerulosa are smaller and more crowded than those in the fasciculata. Mitotic figures are seen in the glomerulosa, the outer fasciculata and the capsule.

7 Female rat, 7 days old. The cells of the glomerulosa are larger than at three days. Mitotic figures occur in the capsule and outer fasciculata.

8 Male rat, 14 days old. The entire cortex is shown, with the edge of the medulla appearing the lower right-hand corner of the picture. Mitotic figures occur in the capsule and throughout the fasciculata. Note particularly the one lying only a few cell layers from the medulla.

9 Male rat, 42 days old. The definitive organization of the adult cortex is illustrated. A pair of telophasic nuclei occurs in the outer fasciculata.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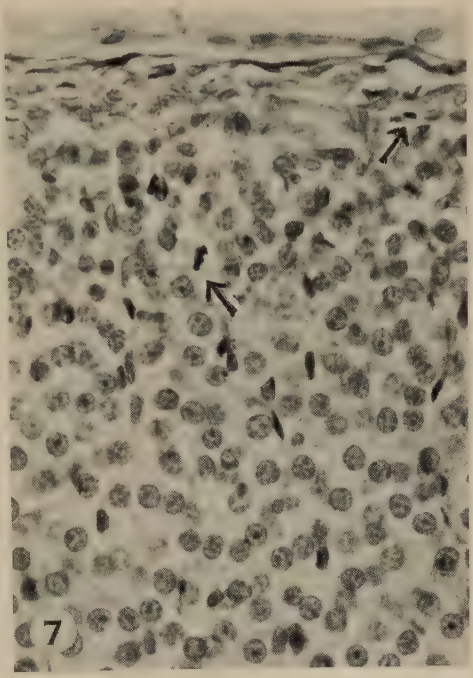
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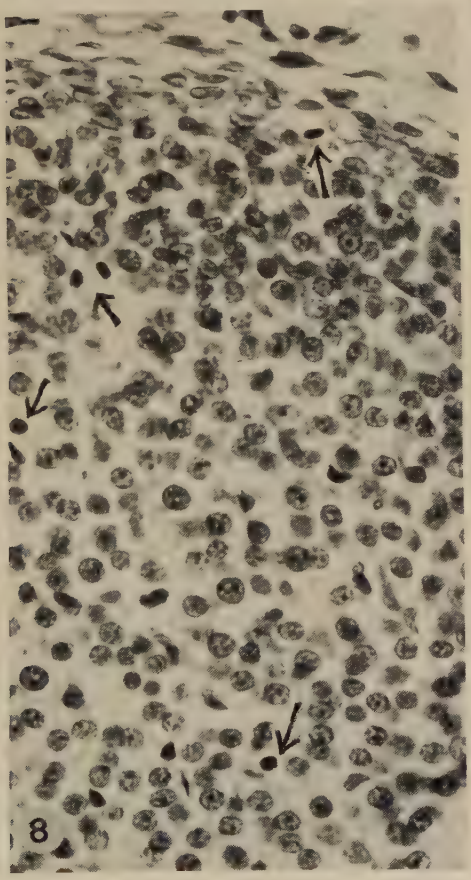
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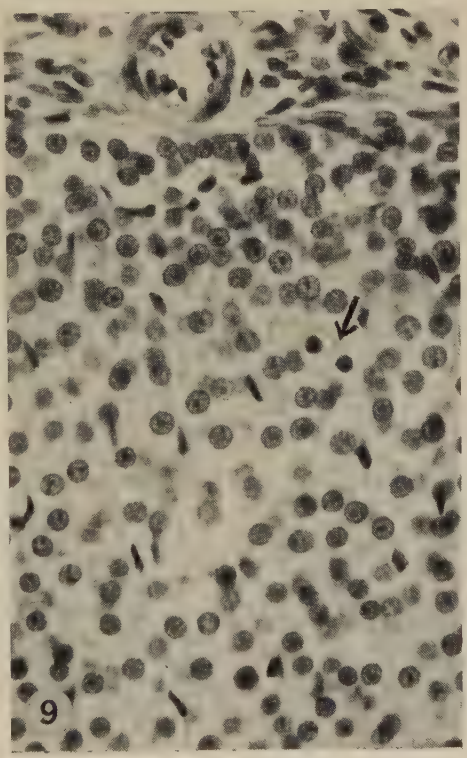
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PLATE 3

EXPLANATION OF FIGURES

Figures 10 to 12 are from adrenal glands that were fixed in Bouin's fluid, sectioned at 10 μ, stained with hematoxylin and eosin, and photographed with green and yellow filters combined (Wratten B and G). Figures 13 to 16 are of adrenal glands that were fixed in 10% formalin and sectioned on the freezing microtome at 15 μ. $\times$ 95.

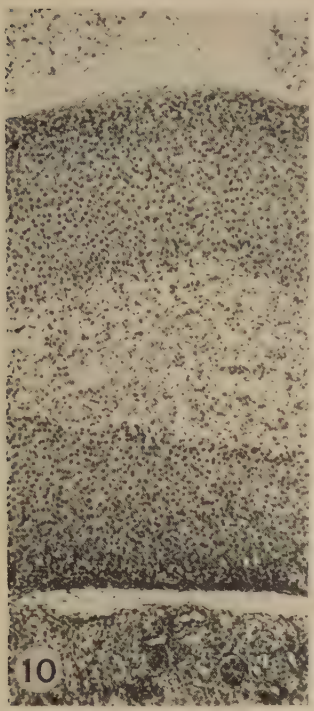
10, 11 and 12 Male rat, 11 days old; female rat, 16 days old; male rat, 42 days old. Compare the width of the cortex at these three ages. By 16 days and more clearly at 42 days, the innermost part of the cortex has acquired a reticulate organization.

13 Female rat, 16 days old. Section stained by the Schultz method for cholesterol. Photographed with a red filter (Wratten F). The droplets in the glomerulosa and outer fasciculata contain material which gives the characteristic blue-green reaction.

14 Male rat, 22 days old. Section stained by the Ashbel-Seligman method for ketonic steroids. Photographed with a green filter (Wratten B). Some of the droplets in the glomerulosa and those in the outer fasciculata stain the positive blue-purple color.

15 Female rat, 16 days old. Untreated section photographed under the polarizing microscope. The outer border of the capsule and the inner border of the cortex have been drawn in. Exceedingly fine birefringent crystals occur throughout the cortex and in the occasional cortical cells lying in the medulla.

16 Male rat, 42 days old. Birefringence picture. The birefringent particles in the glomerulosa are still fine and hence do not show in the photograph. Those in the fasciculata are now mixed in size. Note occasional large crystals in the zona reticularis (compare with fig. 5).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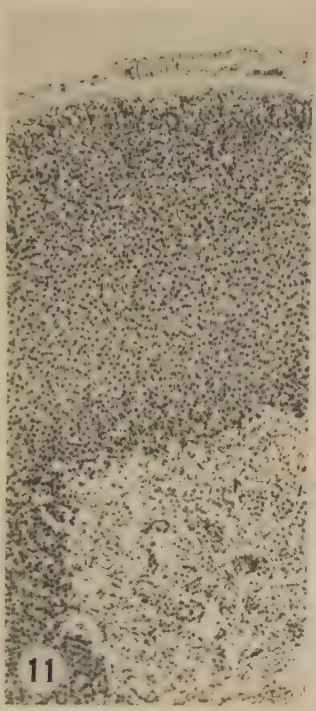
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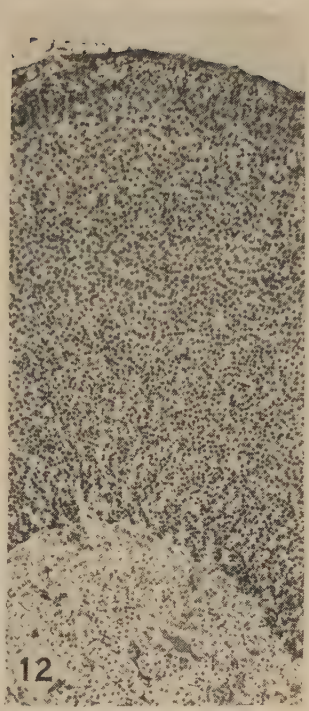
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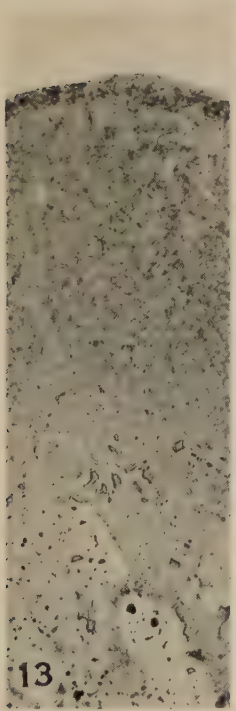
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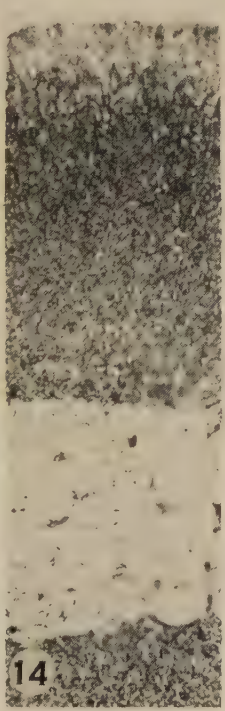
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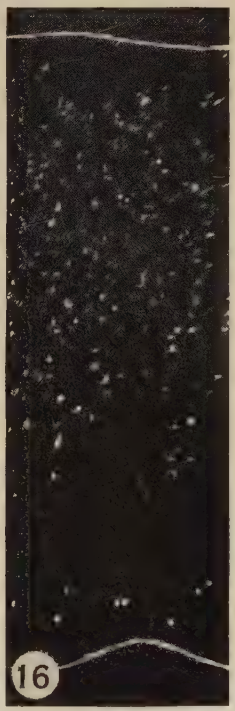
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THE MORPHOLOGY OF THE NERVE CELL NUCLEUS, ACCORDING TO SEX[1]

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TWELVE FIGURES

INTRODUCTION

Cytologists are accustomed to think of sex differences in nuclear morphology in terms of the complement of chromosomes, as seen during cell division. Such studies are difficult in mammalian cells because of the small size and large number of chromosomes. However, the difference in chromosome content of nerve cells in male and female cats leaves its imprint on the "resting" nuclei. An intranuclear body, which appears morphologically as a small satellite to the large nucleolus, is better developed in female than in male cells. We wish to present further details concerning this interesting sex difference, as an elaboration of a preliminary report by Barr and Bertram ('49).

As a temporary convenience, the term "nucleolar satellite" will be used for the sex-influenced body. It is not to be confused with the satellite which occurs at the end of a nucleolar chromosome in some species. As data accumulate, the authors hope that those better informed than ourselves in the field of cytogenetics will come to our assistance in the matter of terminology.

MATERIALS AND METHODS

Twelve mature cats, 6 males and 6 females, were used. Under Nembutal anesthesia, the tissues were fixed with iso-

[1] This investigation was carried out with the aid of grants from the National Research Council of Canada and the National Cancer Institute of Canada.

tonic 10% formalin by perfusion through the vascular system. Blocks were taken from various regions of the nervous system and embedded in paraffin. Sections cut at 12 μ and stained with cresyl violet were used for routine study of nuclear structure, and recording the incidence of the nucleolar satellite and its position within the nucleus; 5 μ sections, also stained with cresyl violet, were prepared for more detailed study and photomicrography. The animals were treated as 6 pairs, each pair consisting of a male and a female. In order to increase the uniformity of staining, sections from the same region from a male and a female cat were mounted on the same slide. In each region, 500 cell sections containing a nucleolus were examined for the presence of a nucleolar satellite. When visible, the position of the satellite was recorded under three general headings — adjacent to the nucleolus, free in the nucleoplasm and adjacent to the nuclear membrane.

In addition to the material from the 12 animals mentioned, sections of nervous tissue from a large number of cats of known sex were examined, though less systematically. These sections from the laboratory collection included material prepared by several technics commonly used in neurohistology.

OBSERVATIONS

Females. The incidence of visible nucleolar satellites in 12 regions of the nervous system is shown in table 1. In order to simplify the table, mean figures for the 6 female cats are recorded. Detailed anatomical localization was not attempted in this initial survey. For example, "hypothalamus" refers to the general hypothalamic area rather than a specific nucleus.

Considering the various regions together, a nucleolar satellite may be identified in three cell sections out of 4. The mammillary bodies were rather difficult to study, the cells being small and unusually susceptible to technical alterations. The relatively low incidence of visible satellites in this region probably does not reflect the true condition.

It is our impression that each nerve cell in the female cat contains a nucleolar satellite. In cell sections in which a satellite cannot be seen, with the cresyl violet stain, it is probably concealed by the large nucleolus or by deeply staining Nissl bodies lying adjacent to the nuclear membrane.

TABLE 1

Incidence and position of nucleolar satellites (%) in 12 μ sections stained with cresyl violet, each cell section containing a nucleolus. Mean of 6 animals of each sex, 500 cells examined in each area in each animal

REGION OF NERVOUS SYSTEM	SEX	POSITION OF NUCLEOLAR SATELLITE (%)			TOTAL (%)
		Adjacent to nucleolus	Free in nucleoplasm	Adjacent to nuclear membrane	
Motor cortex	♀	69.8	5.3	6.3	81.4
	♂	3.2	0.5	0.1	3.8
Purkinje cells	♀	85.7	1.1	0.5	87.3
	♂	4.6	0.6	0.1	5.3
Lateral geniculate body	♀	74.4	3.6	4.4	82.4
	♂	3.2	1.0	0.0	4.2
Hypothalamus	♀	56.8	7.8	11.7	76.3
	♂	4.4	1.8	0.1	6.3
Mammillary body	♀	27.1	3.7	25.3	56.1
	♂	5.4	0.6	0.2	6.2
Hypoglossal nucleus	♀	58.6	10.0	9.0	77.6
	♂	2.2	0.6	0.3	3.1
Dorsal root ganglion (cervical)	♀	39.5	11.1	17.0	67.6
	♂	2.6	1.0	0.4	4.0
Dorsal horn (cervical)	♀	55.2	7.4	11.6	74.2
	♂	3.3	1.8	0.0	5.1
Dorsal horn (lumbosacral)	♀	59.4	7.4	8.1	74.9
	♂	2.6	1.7	0.0	4.3
Ventral horn (cervical)	♀	48.5	13.9	11.6	74.0
	♂	2.7	1.8	0.2	4.7
Ventral horn (lumbosacral)	♀	56.9	12.8	9.0	78.7
	♂	2.3	2.0	0.1	4.4
Stellate ganglion	♀	54.0	9.9	13.6	77.5
	♂	1.5	0.5	0.1	2.1

The position of the nucleolar satellite is also shown in table 1. The satellite is situated adjacent to the nucleolus most frequently (figs. 1, 3, 5, 7 and 10); less often the satellite lies free in the nucleoplasm (fig. 8) or adjacent to the nuclear membrane (fig. 11). The morphological relationship between nucleolus and satellite is especially close in Purkinje cells of the cerebellum. The position of the nucleolar satellite may be altered by experimental conditions which affect the metabolism of the neuron (Barr and Bertram, '49). We are inclined to think that the variations in the position of the satellite under normal conditions may also rest on a functional or metabolic basis. The position pattern of the nucleolar satellite is a further item for consideration in the descriptive morphology of different types of nerve cells.

The nucleolar satellite of the female is spherical or irregular in shape. When adjacent to the nucleolus the satellite may be flattened on its nucleolar side. This is commonly seen in Purkinje cells. A narrow interval between the nucleolus and adjacent satellite is often visible, and the surface of the nucleolus may be flattened (fig. 7) or show a shallow concavity at this point. When the satellite lies against the nuclear membrane it is typically flattened on the membrane side, presenting a triangular or semicircular outline in profile view (fig. 11). Since the satellite changes in shape to conform to adjacent structures, yet retains its individuality as a discrete body, it is probably semisolid in consistency. When the satellite is free of the nucleolus no strands are seen connecting the two structures.

The nucleolar satellite of the female is approximately 1 μ in diameter and, unlike the nucleolus, its volume is remarkably constant from one cell type to another. The nucleolus and satellite were measured in 50 small cells of the dorsal horn and in 50 large cells of the ventral horn of the spinal cord. The average measurements, expressed as volumes, are as follows — dorsal horn cells: nucleolus, 10.5 μ^3, satellite 0.5 μ^3; ventral horn cells: nucleolus, 43.4 μ^3, satellite, 0.5 μ^3.

The position of the satellite appears to bear no relation to the polarity of the neuron. This point was investigated systematically in the hypoglossal nucleus. The axons arise predominantly from the ventromedial aspect of the cell bodies; the satellites have a random spatial orientation.

The satellite, like the nucleolus, stains readily with basic dyes. However, its intensity of staining is variable. The satellite may stain with about the same intensity as the nucleolus, or it may have an increased or decreased affinity for cresyl violet, compared with the nucleolus. Slight variations in staining technic may be responsible, but quantitative or qualitative differences in the chemical composition of the satellite of different cells cannot be excluded. Cells in which the satellite stains more deeply than the nucleolus are most favorable for study. The satellite may then be identified, by careful focussing, even though it is located in the same optical axis as the nucleolus or at the nuclear membrane close to perinuclear Nissl material.

While vacuoles are seen frequently in the nucleolus, no details of internal structure are seen in the nucleolar satellite.

Males. The small size of the nucleolar satellite in male nerve cells prevents satisfactory examination with a standard light microscope. In a small proportion of male neurons a discrete particle is visible, though smaller than the satellite of female cells (table 1, average data for 6 male cats). In many cells the satellite may be just above the limit of resolution but impossible to distinguish from a particle of unrelated chromatin. Because of these difficulties, the figures shown in table 1 are only approximate. They serve the purpose, however, of emphasizing the clear difference in nuclear structure, according to sex. Typical male neurons are illustrated in figures 2, 4, 6, 9 and 12.

The impression that all male neurons contain a nucleolar satellite, though usually too small for accurate identification, is confirmed by certain experimental observations (to be published). Extensive depletion of the Nissl material of hypo-

glossal nerve cells follows prolonged antidromic stimulation. During the recovery phase, in male cells, nucleolar satellites become clearly visible in as high as 38% of cells, although normally the satellite can be identified in less than 5% of cells. Apparently the satellite enlarges during the altered metabolic state of the cell so that a considerable number come to fall within the limits of optical resolution with an oil immersion objective.

Although satellite size differs according to sex, routine examination of the sections conveys the impression that nucleolar volume is the same in males and females. This impression was confirmed by careful measurements in the lateral geniculate body. The average nucleolar volume varies slightly in different animals of the same sex. However, when the values for all of the males are averaged and compared with mean values for all of the females, identical figures result.

Stains other than cresyl violet. The observations which follow apply to nerve cells of female cats. The frequency of visible nucleolar satellites in male neurons remains consistently low regardless of the staining procedure.

(a) *Thionin.* This stain, applied to celloidon sections, is especially useful because of its metachromatic properties. The Nissl material and nucleolus stain violet; the nucleolar satellite stains blue. This differential staining facilitates identification of the satellite even when it lies in the same optical axis as the nucleolus or at the periphery of the nucleus and close to masses of Nissl material. More accurate information concerning the incidence and position pattern of the satellite can be obtained with thionin than with cresyl violet. Cells of the lateral geniculate body in 6 female cats were examined systematically after celloidon embedding and staining with thionin (14 μ sections). The mean figures are as follows — adjacent to nucleolus, 77.2%; free in nucleoplasm, 2.1%; adjacent to nuclear membrane, 16.7%; total, 96.0%.

(b) *Feulgen and methyl green-pyronin technics.* These methods are valuable for their cytochemical properties. The nucleolar satellite is consistently Feulgen-positive, while the

nucleolus and Nissl material are Feulgen-negative. With methyl green-pyronin the satellite stains green; the nucleolus and Nissl material stain red. These observations, combined with data on the cytochemistry of the neuron now in the literature (Hydén, '47), indicate that the nucleolar satellite contains desoxyribose nucleic acid, the nucleolus and Nissl material ribose nucleic acid.

(c) *Silver methods and iron hematoxylin.* The nucleolar satellite is well stained by Bodian's protargol method, various modifications of Cajal's reduced silver nitrate method and by iron hematoxylin. For the present purpose, the latter methods have the disadvantage of staining considerable intranuclear material in addition to the nucleolus and its satellite.

Human nerve cells. It is our intention to restrict this report almost exclusively to a description of the nerve cell nucleus of the cat. However, in the section to follow, there will be occasion to refer to the nucleolar chromosomes of man. Certain general statements on nuclear morphology in the human neuron are, therefore, required. Detailed observations on human material will be reported later.

Human sympathetic ganglion cells show the same consistent sex difference as occurs throughout the nervous system of the cat. In female cells, the satellite is situated frequently at the nuclear membrane where it may be flattened in the form of an exceedingly thin disc. The nucleolus is often eccentric in the same direction, the satellite lying between nucleolus and nuclear membrane and touching both. Nucleolar satellites are seldom encountered in male sympathetic ganglion cells and, when visible, are very small.

Human material, however, shows interesting variations in nuclear morphology according to the cell type. There is very little sex difference in Purkinje cells. Nucleolar satellites are visible with about the same frequency in male and female Purkinje cells but are, on the whole, a little smaller in the male. The difference is insufficient to permit identification of the sex of the individual from whom a section of cerebellar cortex was obtained. Pyramidal cells of the cerebral cortex

(area 4) lie between the extremes presented by sympathetic ganglion and Purkinje cells. Our preliminary study of human material suggests that nuclear morphology, from the standpoint of sex difference, is similar in the human and the cat but that the nucleolar satellite of the male human enlarges in certain types of neurons, possibly as a result of functional demands made upon it.

DISCUSSION

The basis for a sex difference in the morphology of the resting nucleus is a question of compelling interest. In the absence of direct evidence, the brief discussion which follows must be regarded as exploratory and subject to extensive modification as more facts become available.

According to de Winiwarter ('20), the chromosome complement for the cat is 34 + XX for the female and 34 + XO for the male. Our reasoning is based on the assumption that the difference in number of X chromosomes is the primary factor which determines the distinction between a female and male resting nucleus.

Insects and plants are the main source of data concerning chromosome morphology. In these forms the X chromosome is often the nucleolar chromosome (Gates, '42). Accordingly, Barr and Bertram ('49) suggested that the nucleolar satellite may be derived from that portion of the nucleolar chromosome known as the nucleolar organizer, the female having a double complement of this material, compared with the male. This interpretation now appears unlikely.

The nucleolus and the nucleolar satellite may not be as intimately related as the morphological appearance of the resting nucleus suggests. Schultz and St. Lawrence ('49), in an important paper, have shown that the nucleolar chromosome of man is an autosome. The formation of the nucleolus in man should not be influenced by the sex of the individual, since it arises from a pair of autosomes in both males and females. The equal nucleolar volume in male and female cats could be regarded as a point in favor of a similar origin of the nucleolus in the cat. Schultz and St. Lawrence have also

recorded the significant observation that, in man, "a nucleolus is formed by the X chromosome, independent of that formed by the nucleolar chromosome." It is possible that a similar phenomenon occurs in the cat, the body formed by the X chromosome becoming the nucleolar satellite of the resting nucleus. If this is the case, the body formed by the single X chromosome of the male is so small that it is seen with difficulty in the resting nucleus. In the female, however, two small masses of X chromosome origin would be available to fuse and form a nucleolar satellite of appreciable size. It is to be hoped that workers familiar with the technics of cytogenetics will be able to provide data for the cat and other mammals similar to that recorded by Schultz and St. Lawrence for man.

The knowledge that a nuclear component differs in size according to sex may be expected to serve as a stimulus to inquiry into its function in cell metabolism. Experiments in this laboratory clearly indicate that the nucleolar satellites of male and female neurons enlarge during the recovery of a cell from chromatolysis induced by prolonged antidromic stimulation (Bertram et al., '50). There is a concurrent enlargement and increased vacuolation of the nucleolus. According to Hydén ('47), the nucleolus participates in the synthesis of ribose nucleoproteins of Nissl material. The enlargement of the nucleolar satellite during accelerated formation of Nissl material suggests that the satellite, as well as the nucleolus, may be concerned with nucleoprotein synthesis. However that may be, it is interesting to speculate that some aspect of nerve cell metabolism may eventually prove to be different quantitatively in the two sexes, in favor of the female.

SUMMARY

1. A difference in the morphology of the nerve cell nucleus of the cat, according to sex, is described. In the female, a body about 1 μ in diameter appears as a small satellite to the large nucleolus in all types of nerve cells examined. In the male, a nucleolar satellite is seldom seen distinctly. There is evidence

that male nerve cells contain a nucleolar satellite so small that it lies at the limit of resolution with standard optical equipment.

2. The results of staining with Feulgen and methyl green-pyronin methods indicate that the nucleolar satellite contains desoxyribose nucleic acid, unlike the nucleolus which contains ribose nucleic acid.

3. On the basis of indirect evidence, it is suggested that the nucleolar satellite is a product of the X chromosome. From the cytogenetic point of view, the satellite may be less intimately related to the nucleolus than the morphological appearance of the resting nucleus would suggest.

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PLATES

All illustrations are of nerve cells of the cat, prepared from 5 μ sections stained with cresyl violet, magnification $\times$ 1200.

PLATE 1

EXPLANATION OF FIGURES

1 Purkinje cell, female. The well defined satellite body, typical of female cells, is situated adjacent to the nucleolus. The morphological relation between the nucleolus and its satellite is especially close in Purkinje cells.

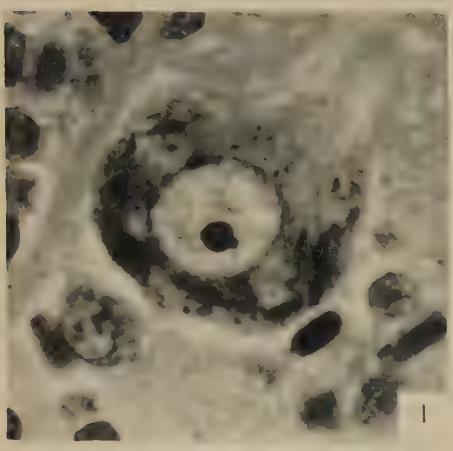
2 Purkinje cell, male. No nucleolar satellite is visible.

3 Motor neuron of the spinal cord, female, with typical satellite located adjacent to the nucleolus.

4 Motor neuron of the spinal cord, male. A nucleolar satellite is not visible.

5 Stellate ganglion cell, female, showing the characteristic female nuclear morphology.

6 Stellate ganglion cell, male, lacking a visible nucleolar satellite.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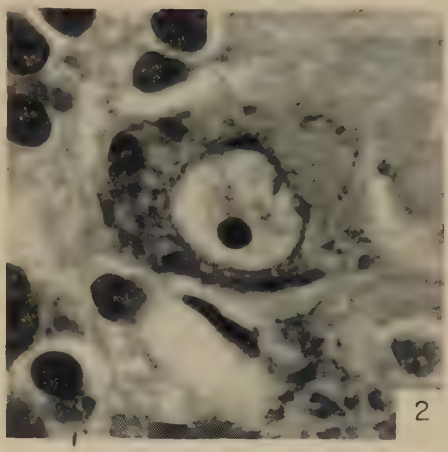
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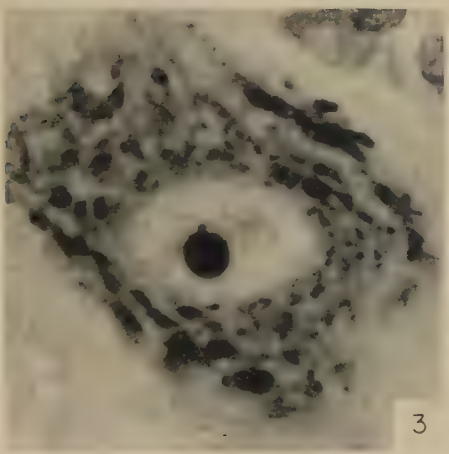
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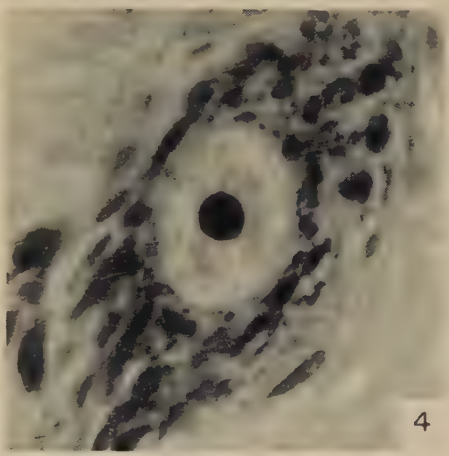
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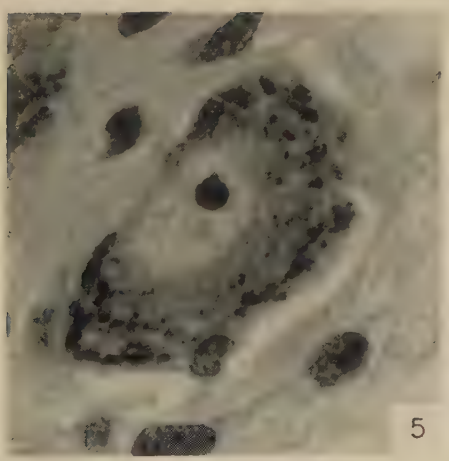
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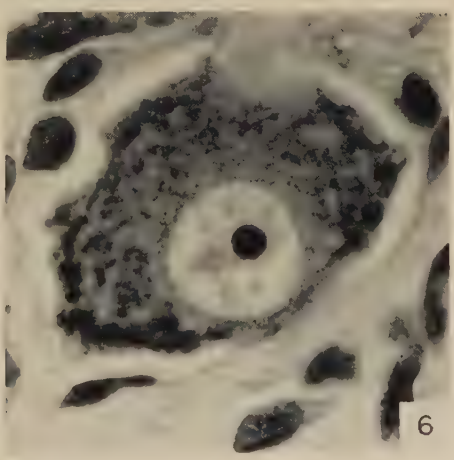
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PLATE 2

EXPLANATION OF FIGURES

7 Betz cell of motor cortex, female, showing a satellite adjacent to the nucleolus.

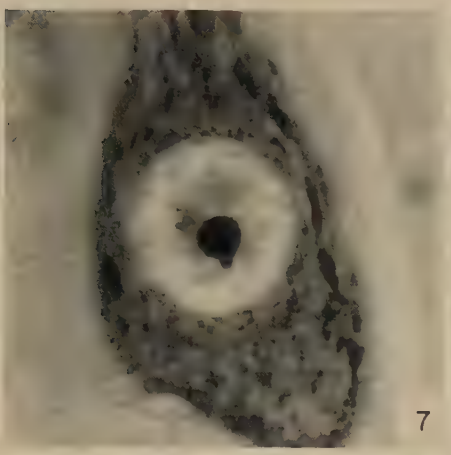
8 Betz cell of motor cortex, female, to illustrate a nucleolar satellite free in the nucleoplasm.

9 Betz cell of motor cortex, male, with no visible nucleolar satellite.

10 Dorsal root ganglion cell, female, illustrating typical female nuclear morphology.

11 Dorsal root ganglion cell, female. The nucleolar satellite lies against the nuclear membrane.

12 Dorsal root ganglion cell, male. There is no visible nucleolar satellite.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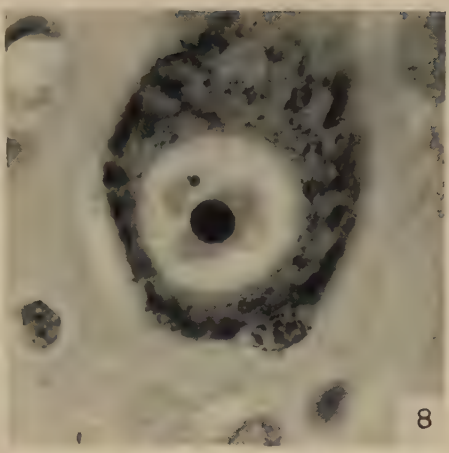
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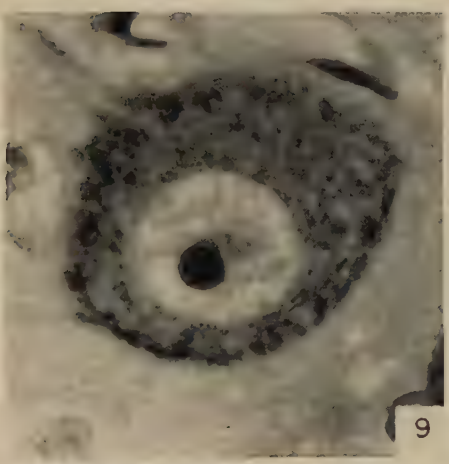
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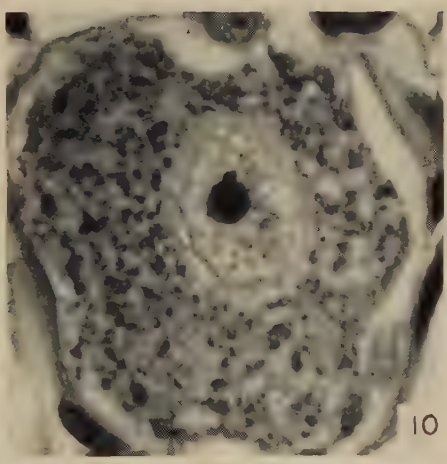
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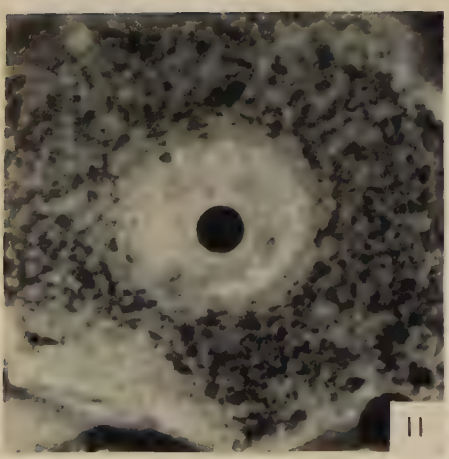
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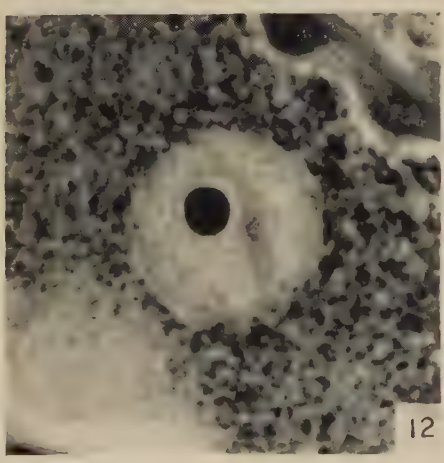
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RESPONSE BY THE RAT THYRO-PARATHYROIDECTOMIZED AT BIRTH TO GROWTH HORMONE AND TO THYROXIN GIVEN SEPARATELY OR IN COMBINATION [1]

III. SKELETAL CHANGES: TIBIA, METACARPAL, AND CAUDAL VERTEBRAE

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THIRTY FIGURES

Two recent reports have dealt with the response of rats thyroidectomized at birth to administration of pituitary growth hormone and thyroxin. The body growth and organ changes were described by Scow et al., '49. Although such rats made substantial gains in body weight and length when treated with pure growth hormone, they remained immature, as did their uninjected thyroidectomized controls, judged both from external appearances and from roentgenograms of the skeleton. On the other hand, when treated with thyroxin, the animals not only grew markedly, but also advanced in maturity, somatic and skeletal. A second report by Koneff et al., '49, demonstrated that the administration of thyroxin repaired the pituitary cytologic deficiencies which had attended

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early thyroidectomy. In particular, the eosinophils (the putative source of growth hormone) were restored to normal. As anticipated, administration of growth hormone did not correct the pituitary pathology. It thus appeared that the excellent balance between growth and maturation shown by the thyroxin-treated rats was due as much to the re-establishment of an endogenous supply of growth hormone as to the direct effect of thyroxin itself. Thyroidectomized rats injected with both of these hormones made gains comparable to, but not substantially in excess of, such gains made by rats injected with thyroxin alone. This suggested that the optimum had been achieved by the thyroxin injections and that additional, exogenous supplies of growth hormone did not appreciably increase the growth rate of these already rapidly growing rats.

It is the purpose of this report, the third in the series, to describe the histologic differences seen in representative skeletal structures of these rats. Special attention was directed in this study to evidences of skeletal maturity, particularly to presence and extent of development of ossification centers. The objective was in part to correlate histologic structure with the roentgenographic diagnoses of "bone age." It was also of importance to seek evidence of imbalance in endochondral ossification which would account for skeletal growth without differentiation in the growth-hormone injected rats, as contrasted with the balanced growth and differentiation in those injected with thyroxin.

The conditions of the experiment have been described in detail in the first report of this series. Briefly, they were as follows: Approximately 300 rats of the Long-Evans strain were thyro-parathyroidectomized at birth. Of these, 60 were selected when 30 days of age as being presumptively completely thyroidless. The basis for this choice was evidence of marked delay in development — pellage, ears, external genitalia, and skeleton. The last seemed most critical, and no rats were included whose roentgenographic "bone age" exceeded 15 days at a chronologic age of 30 days. The 60 rats

thus chosen were divided into 4 groups of 15 rats each. The first group received pure anterior hypophyseal growth hormone, 0.5 mg daily, injected intraperitoneally, for 30 days. Each dose contained 25 rat units of growth hormone, as assayed in hypophysectomized immature female rats (Li, Evans and Simpson, '45). The second group received crystalline thyroxin (Squibb), 2.5 μg daily, injected subcutaneously for a like period. A third group received both hormones in the dosage specified. The 4th group was retained as uninjected thyroidectomized controls. Two groups of normal rats were sacrificed as controls. One group (20 rats) was 61–64 days of age at autopsy, to correspond in *chronologic age* to the experimental animals at the time of autopsy. The other group (12 rats) was 18 days of age at autopsy; these rats were designated *bone age* controls, since they corresponded closely in size and skeletal immaturity to the uninjected thyroidectomized rats autopsied at 60 days of age.

At autopsy the completeness of thyroidectomy was verified by the radioautographic technique of Reinhardt ('42). After rejection of those showing possibly incomplete thyroidectomy, there remained for study 6 rats injected with growth hormone, 11 with thyroxin, 10 with both hormones, and 6 uninjected thyroidectomized controls. Both sexes were represented in every group.[3]

After formol fixation and roentgenography of the carcasses in an extended position, representative portions of the skeleton were removed and prepared for histological study. These included the proximal epiphysis of the tibia, the third meta-

[3] The major attention of the present study is to the findings on the females, since in this sex more extensive histological skeletal data have been accumulated for normal standards. Males are not illustrated, and are described only when they differed markedly from the females in skeletal structure. Sex differences are probably unimportant in the uninjected thyroidectomized controls and the growth hormone-injected rats, judged from the immaturity of their external and internal genitalia. However, marked genital development and evidences of function were seen in the rats receiving thyroxin, as a consequence of pituitary repair and resumption of gonadotrophin output, and sex distinction is therefore necessary.

carpal, and the 3rd, 8th, and 13th caudal vertebrae. The mandibular condyle, the molar teeth, and the upper central incisors are being described in a subsequent report.

RESULTS

Tibia

The proximal epiphysis of the tibia, its cartilage plate, and the neighboring portion of the tibial diaphysis were examined to determine their size, degree of development, and endochondral ossification activity.

The bone structure of the normal controls, both 18-day-old bone-age controls and 61–64-day-old autopsy controls is shown in figures 1 and 2. The differences in the expansion of the epiphyseal ossification center at these two ages afford a basis for estimating the maturity attained by this bone in the various experimental groups. In the 18-day-old rats the ossification center occupied 26% of the cartilaginous epiphysis, while in the 61–64-day-old rats, it had expanded to become 71% of the epiphysis.[4]

In the thyroidectomized controls (fig. 3) the epiphyseal ossification center was small and had expanded only slightly into the cartilaginous epiphysis. In per cent of total epiphyseal area (40%) it only slightly exceeded the tibia of the young bone-age controls, lending confirmation to the 18-day-bone age diagnosed roentgenographically in these rats. However, the thyroidectomized rats showed a narrower epiphyseal cartilage plate than the 18-day-old normal rats.

The cells were smaller and the matrix more abundant. There was evidence of bone formation on the entire periphery of the epiphyseal ossification center but there appeared to be little erosion of the surrounding cartilage. Next the diaphyseal marrow junction the cartilage cells were enlarged but not vacuolated. The bony trabeculae were sparse and short. The marrow was cellular and its sinusoids so patent that it was liver-like. The cortical bone of the diaphysis was extremely thin.

[4] The areas occupied by the cartilaginous epiphysis and the ossification centers within them were traced and measured with a planimeter.

In the growth hormone-injected rats (fig. 4) the tibia itself was substantially larger but the epiphyseal ossification center did not expand, and remained of the size of the 18-day-old normal rats. It occupied 25% of the cartilaginous epiphysis. This added confirmation to the conclusion, based on roentgenograms, that the administration of growth hormone to these thyroidectomized rats did not advance the bone age.

At the periphery of the ossification center marrow tufts were present. The entire cartilaginous part of the epiphysis was proliferating. The epiphyseal cartilage plate was more clearly divided into zones than was the thyroidectomized control and showed clearly the delimitation of the future disc. In particular the cells next the diaphyseal marrow junction were enlarged, vacuolated, and subject to active erosion. The marrow cavity was filled with delicate bony trabeculae. The cortical bone was somewhat thicker than in the controls and was very vascular.

In the rats injected with thyroxin (fig. 5) the tibias were bigger than in the preceding groups. The development of the epiphyseal ossification center was complete, and bone sealed its marrow from the surrounding cartilage. The proportion of the epiphysis occupied by this tissue (55%) approached that in the 61–64-day-old normal rats. A true epiphyseal plate had been established.

The plate was very cellular, the majority of chondrocytes being in columns. At the marrow junction there was active erosion and innumerable delicate bony trabeculae extended far into the marrow. They remained parallel even to the secondary spongiosa. The cortical bone of the diaphysis was substantially thicker than in the controls or growth hormone treated rats. The marrow was cellular.

In the rats treated with both growth hormone and thyroxin (fig. 6) the bones were no bigger than the thyroxin-treated group, and resembled them in differentiation. Bone and marrow constituted 68% of the epiphyseal area. They were scarcely distinguishable from normal controls of the same chronologic age except for a wider epiphyseal cartilage plate. This supported the bone-age diagnosis of marked advance in skeletal maturity during treatment with thyroxin.

Metacarpal

Figures 7–12 show that the response of the metacarpal was essentially the same as that of the tibia. In particular figure 9 shows the retarded development of the thyroidectomized controls. Figure 10 shows the excessive cartilage formation resulting from growth hormone injections. Figures 11 and 12 show well the aging which took place when the rats were injected with thyroxin, with or without growth hormone. The cellularity of the marrow in these last two groups was noteworthy.

Caudal vertebrae

The 3rd, 8th, and 13th caudal vertebrae were studied histologically for two distinct purposes. First, information was sought as to whether these representatives of the axial skeleton responded to the various experimental regimens in the same fashion as did appendicular bones. Second, since the degree of development of the caudal epiphyseal ossification centers, as shown in roentgenograms, had been one of the critical factors in diagnosing the skeletal age attained by rats of the different groups, it was especially important here to attempt histological correlations with the roentgenographic findings.

The histologic examination showed that the caudal vertebrae had responded in a manner essentially similar to that described for the tibia and metacarpal. The normal standards for the different vertebral levels in 18-day-old and 61–64-day-old controls are shown in figures 13–15 and 16–18, respectively. The vertebrae of the thyroidectomized controls (figs. 19–21) corresponded in size to those of the 18-day-old "bone-age" controls but were less mature. Although the diaphyses were osseous, the epiphyses were predominantly cartilaginous. Only the cartilage basophilia and vacuolation which adumbrates the appearance of an ossification center could be found. The growth-hormone injected thyroidectomized rats had caudal vertebrae substantially larger, though they remained immature as in the thyroidectomized controls (figs.

22–24). The orderly arrangement of cartilage cells was a conspicuous feature. The rats injected with thyroxin, whether alone (figs. 25–27) or in combination with growth hormone (figs. 28–30) had caudal vertebrae which in size and differentiation approached those of the normal 61–64-day-old controls. The secondary epiphyseal ossification centers were fully expanded and osseous. They were separated from the diaphysis by cartilage plates whose structure indicated active endochondral osteogenesis.

Efforts at correlating histological and roentgenographic findings in the caudal vertebrae were especially instructive in determining the interpretations that could be made from the roentgenographic findings. The discrepancies were not marked enough to invalidate the roentgenographic technique of skeletal age determinations. However, it was not always possible to confirm histologically the roentgenographic diagnosis of the initial establishment of an ossification center. Although radio-opaque areas suggesting small epiphyseal ossification centers were found, the histologic studies often showed only cartilage basophilia (presumptive calcification). Some histological sections showed small epiphyseal ossification centers which did not appear in the roentgenograms. For example, although in 18-day-old normal rats epiphyseal ossification centers were found histologically at both ends of the 8th and 13th caudal vertebrae, no corresponding radio-opaque areas could be detected. Roentgenographic diagnoses of "fusion" of epiphyses with diaphyses of caudal vertebrae were not sustained, since intact cartilage plates were always found on histological examination. However, such plates were often so narrow and lacking in flatness that there was no straight course through which the roentgen rays could pass to cast the image of the plate. Thus, although differences in the 3rd, 8th, and 13th caudal vertebrae had been anticipated on the basis of the roentgenographic findings, they were not sustained by histological study, as shown by the photomicrographs of these three levels in the various groups.

SUMMARY

The skeletal development in rats thyroidectomized at birth and injected from 30 to 60 days of age with growth hormone, thyroxin, or the two combined has been studied histologically at the proximal epiphysis of the tibia, the third metacarpal, and the 3rd, 8th, and 13th caudal vertebrae. Histologic analysis of growth and differentiation was compared with roentgenographic analysis of these animals. There was clear confirmation that the thyroidectomy markedly retarded growth and differentiation, that growth hormone stimulated growth without differentiation, and that thyroxin stimulated both growth and differentiation. No augmentation of either of these effects was seen when the two hormones were administered together. Although the histologic findings generally confirmed the roentgenographic diagnoses, some discrepancies were found. The roentgenograms did not always show the beginnings of secondary ossification centers, nor in them could bony centers be distinguished from calcified cartilage. Also, roentgenographic diagnoses of epiphyseal "fusion" of caudal vertebrae were not sustained histologically. These discrepancies, however, were not so marked as to invalidate the roentgenographic technique of skeletal age determinations.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

Photomicrographs of central sagittal sections of the proximal epiphyseal region of the tibia of rats. H. and E. stain. × 10.

1 Normal "bone-age" control, 18 days of age at autopsy. Spec. 9285, Pl. B482.

2 Normal control, 63 days of age at autopsy. Spec. 9305, Pl. B483.

3 Thyroidectomized control, 62 days of age at autopsy; thyroidectomized at one day of age. Spec. 9251, Pl. B484.

4 Growth hormone, 0.5 mg daily for 30 days, beginning at 32 days of age, thyroidectomized at one day of age. Spec. 9252, Pl. B485.

5 Thyroxin, 0.0025 mg daily for 30 days, beginning at 32 days of age; thyroidectomized at one day of age. Spec. 9259, Pl B490.

6 Growth hormone and thyroxin, each given as above, beginning at 33 days of age; thyroidectomized at one day of age. Spec. 9262, Pl. B493.

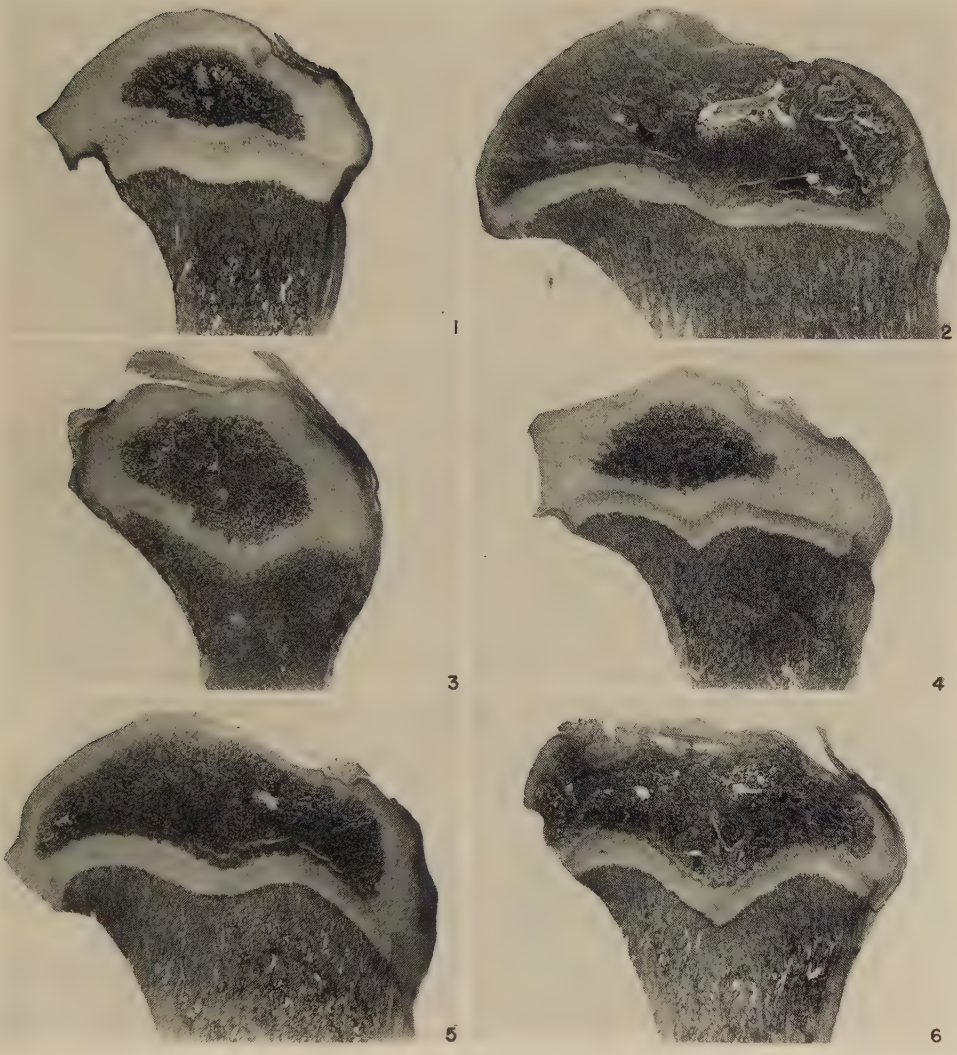
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PLATE 2

EXPLANATION OF FIGURES

Photomicrographs of the epiphyseal region of the third metacarpal of rats. Median sagittal sections, H. and E. × 24.

7 Normal bone-age control, 18 days of age at autopsy. Spec. 9283, Pl. B531.

8 Normal control, 61 days of age at autopsy. Spec. 9302, Pl. B532.

9 Thyroidectomized control, 62 days of age at autopsy, thyroidectomized at one day of age. Spec. 9251, Pl. B533.

10 Growth hormone, 0.5 mg daily for 30 days, beginning at 33 days of age; thyroidectomized at one day of age. Spec. 9330, Pl. B534.

11 Thyroxin, 0.0025 mg daily for 30 days, beginning at 31 days of age, thyroidectomized at one day of age. Spec. 9256, Pl. B535.

12 Growth hormone and thyroxin, each given as above, beginning at 34 days of age; thyroidectomized at one day of age. Spec. 9353, Pl. B536.

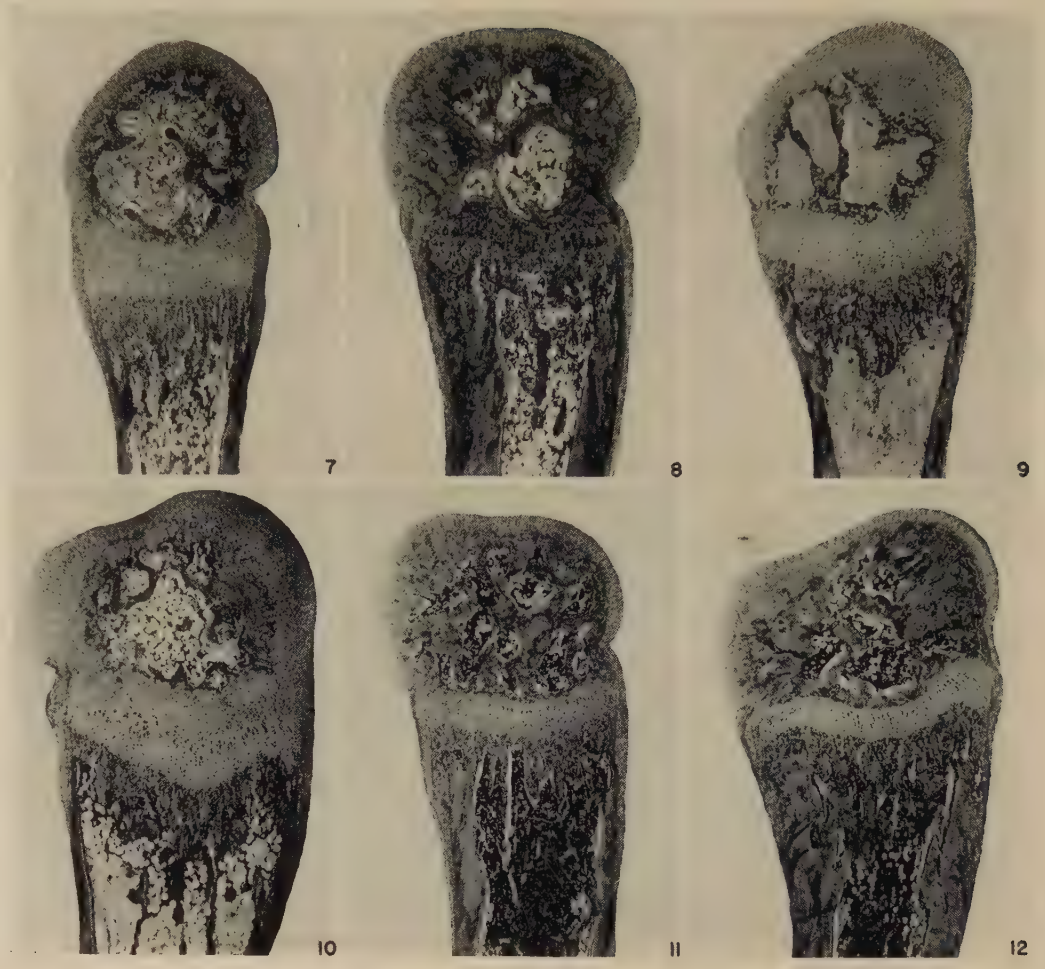
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PLATE 3

EXPLANATION OF FIGURES

Photomicrographs of the epiphyseal region of the 3rd, 8th and 13th caudal vertebrae of normal rats. Median sagittal sections, H. and E. × 17.

Normal bone age control, 18 days of age at autopsy. Spec. 9284.

13 Third caudal vertebra. Pl. B555.
14 Eighth caudal vertebra. Pl. B556.
15 Thirteenth caudal vertebra. Pl. B557.

Normal control 63 days of age at autopsy. Spec. 9295.

16 Third caudal vertebra. Pl. B558.
17 Eighth caudal vertebra. Pl. B559.
18 Thirteenth caudal vertebra. Pl. B560.

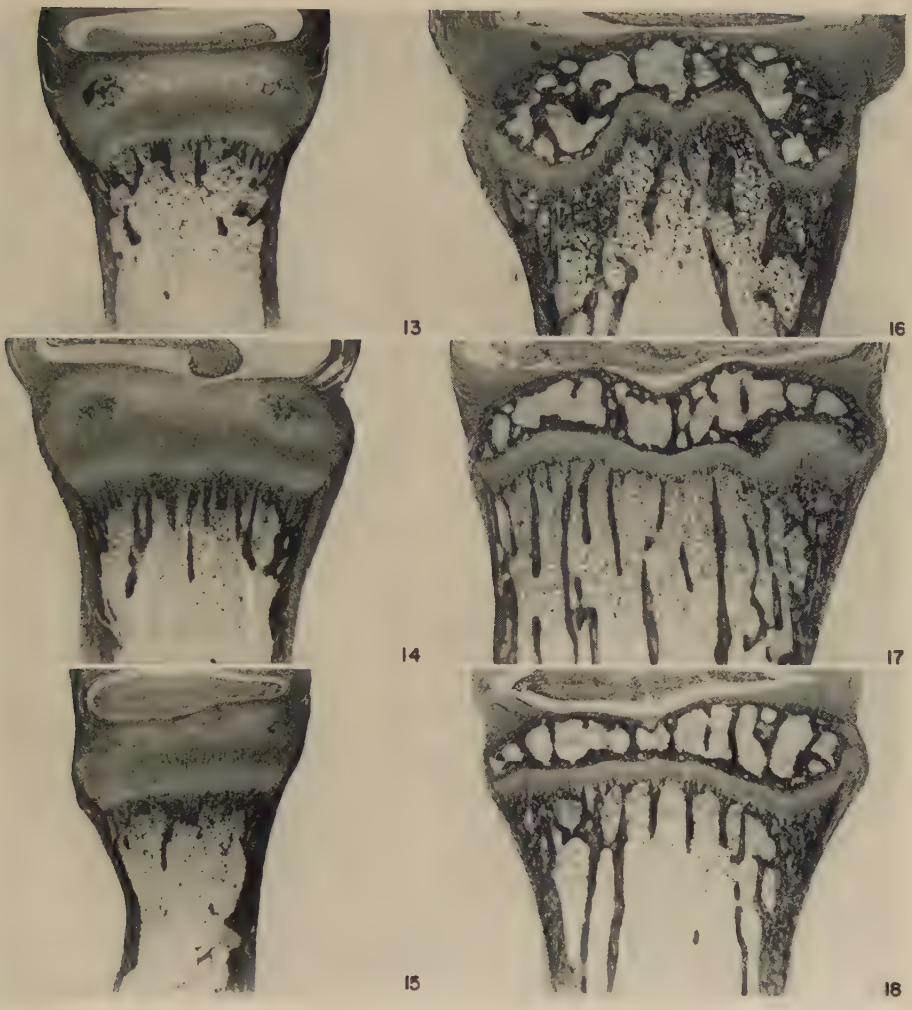
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PLATE 4

EXPLANATION OF FIGURES

Photomicrographs of the epiphyseal region of the 3rd, 8th and 13th caudal vertebrae of rats thyroidectomized at one day of age. Median sagittal sections, H. and E. × 17.

Thyroidectomized control, 62 days of age at autopsy. Spec. 9251.

19 Third caudal vertebra. Pl. B561.
20 Eighth caudal vertebra. Pl. B562.
21 Thirteenth caudal vertebra. Pl. B563.

Growth hormone, 0.5 mg daily for 30 days, beginning at 32 days of age. Spec. 9252.

22 Third caudal vertebra. Pl. B564.
23 Eighth caudal vertebra. Pl. B565.
24 Thirteenth caudal vertebra. Pl. B566.

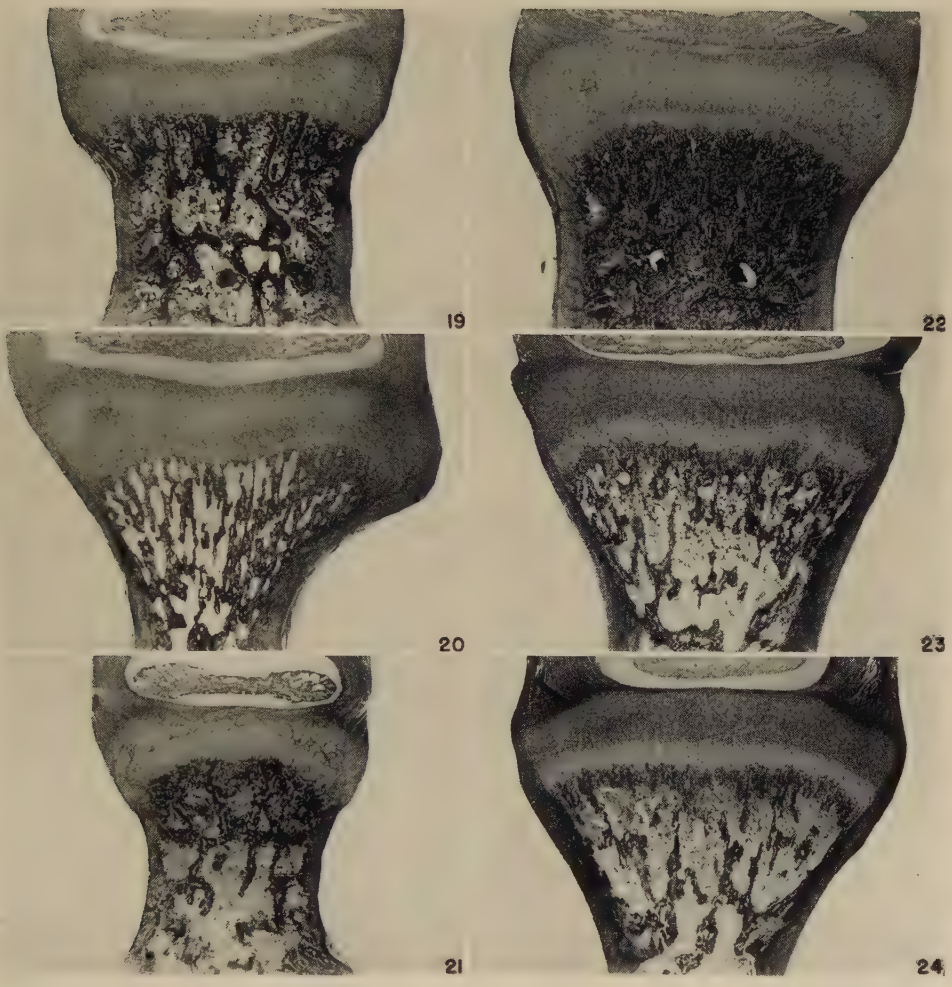
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PLATE 5

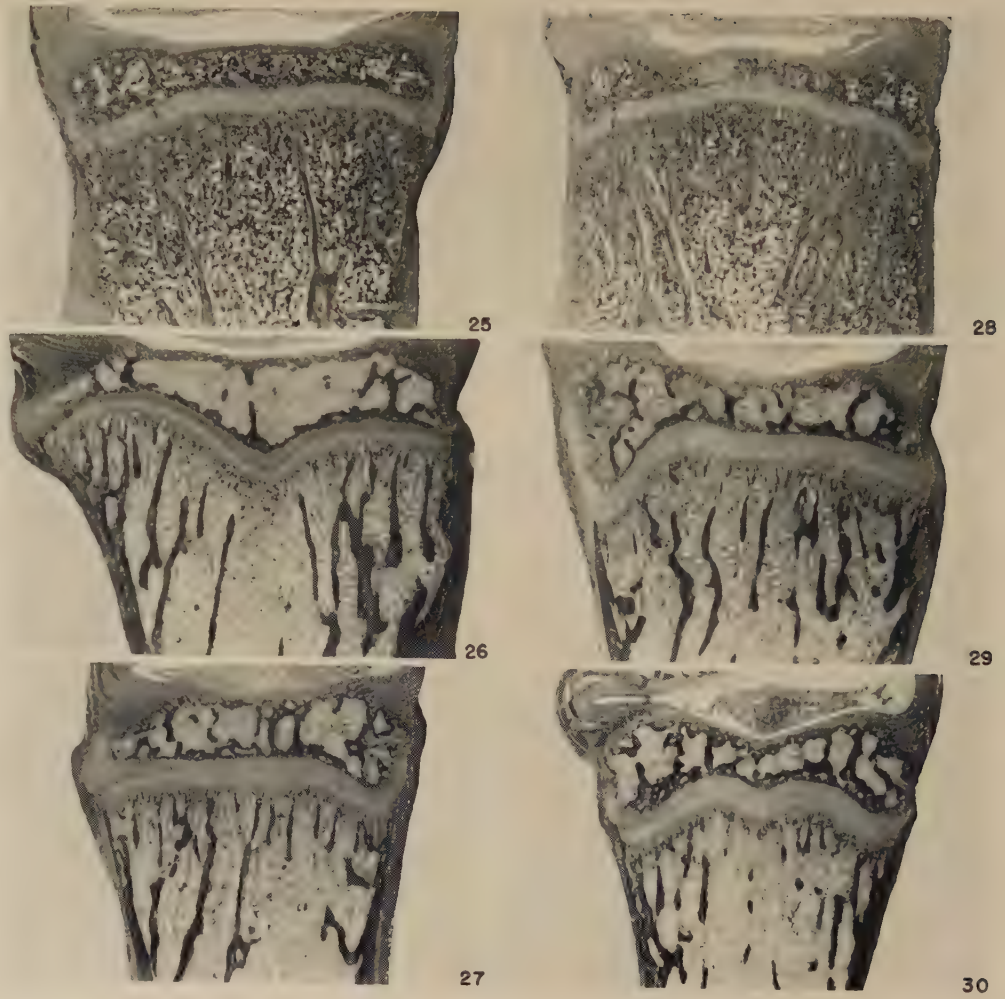
EXPLANATION OF FIGURES

Photomicrographs of the epiphyseal region of the third, 8th and 13th caudal vertebrae of rats thyroidectomized at one day of age. Median sagittal sections, H. and E. × 17. Thyroxin, 0.0025 mg daily for 30 days, beginning at 34 days of age. Spec. 9339.

25 Third caudal vertebra. Pl. B567.
26 Eighth caudal vertebra. Pl. B568.
27 Thirteenth caudal vertebra. Pl. B569.

Growth hormone, 0.5 mg, and thyroxin, 0.0025 mg, daily for 30 days, beginning at 34 days of age. Spec. 9353.

28 Third caudal vertebra. Pl. B570.
29 Eighth caudal vertebra. Pl. B571.
30 Thirteenth caudal vertebra. Pl. B572.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A TECHNIQUE FOR THE DEMONSTRATION OF THE BLOOD VESSELS IN THE DEVELOPING CENTRAL NERVOUS SYSTEM

ERNST SCHARRER

Department of Anatomy, University of Colorado School of Medicine, Denver, Colorado [1]

FOUR FIGURES

The technical difficulties involved in attempts to demonstrate blood vessels in embryos and fetuses constitute an important limiting factor in the investigation of the development of the cerebral vascular system.

The methods available for the demonstration of blood vessels are based either on the injection of the vessels or on the staining of their content of red blood corpuscles. The injection of blood vessels may be accomplished in two ways. The heart may be used in the living animal to distribute throughout the vascular system a non-toxic dye such as chlorazol sky blue (Campbell, '38) or a colloid solution of mercuric sulfide (Daron, '36). These methods have not been tried by investigators interested in the development of the blood vessels of the central nervous system. More commonly the blood vessels are injected with colored gelatine, latex, etc., under pressure, applied by means of a syringe or a percolator, after the animals have been perfused with saline to remove the blood. These methods, although useful in many instances, have a number of disadvantages which are discussed by Drummond ('44). Quantitative studies, in particular of the pre- and postnatal changes in cerebral vascularity, require complete in-

[1] This paper reports research undertaken under contract with the Office of Naval Research. The conclusions contained in this report are those of the author and do not necessarily reflect the views of the Office of Naval Research.

jections which, as Craigie ('25) and Petrén ('38) point out, are difficult to obtain in mammalian embryos and fetuses. Injections with India ink have been successfully practiced by Feeney and Watterson ('46) in chick embryos.

The second type of technique, namely the staining of red cells in the capillaries, has been applied to the blood vessels of the brain by several investigators. The benzidine-nitroprusside method for hemoglobin of Pickworth ('34) and the similar method of Sjöstrand ('34) depend on the uniform filling of the capillaries with red blood corpuscles at the time of death. In order to cause dilation of the capillaries and thus assure their being filled with erythrocytes, Petrén ('38) kills the animals by intracardial injection of histamine. Even if it should be possible to achieve uniform filling of the cerebral capillaries by this or similar methods, the subsequent handling of the tissue and loss of blood during removal of the brain is likely to disturb the distribution of the red cells in the cerebral vascular bed. These methods furthermore require frozen sections, the preparations are said not to be permanent, and precipitates around the vessels may disturb the picture. Not all these objections apply to the methods of Altschul ('39) and Eros ('41) who stain the contents of the blood vessels with acid fuchsin in either paraffin, celloidin or frozen sections.

It appears from this brief review that none of these methods is quite satisfactory for the study of the development of the blood vessels of the central nervous system. A new approach was suggested by the observation of Landow, Kabat and Newman ('42) who reported the presence in the adult central nervous system of alkaline phosphatase in the cells of the vascular endothelium. The possibility was explored, therefore, of "staining" selectively the blood vessels of the fetal brain and spinal cord by a method that demonstrates alkaline phosphatase in the endothelium.

MATERIAL AND METHODS

Gomori's ('39) method for the demonstration of alkaline phosphatase in tissues was used in this study. Since we were

interested in the application of this technique as a "staining" method, it was necessary to standardize it so that it could be used in the laboratory as a routine staining method rather than as a histochemical procedure.[2]

A number of embryos and fetuses of rats and pigs were removed from the uteri and, if small enough, fixed in toto in chilled absolute alcohol. In the case of older specimens the skull was removed in order to permit the effective penetration of the fixing fluid; in still older stages pieces of the brain or the spinal cord were fixed. These pieces were not thicker than 3 or 4 mm in order to assure adequate fixation in the central portions. Apparently one need not be too much concerned in this respect. We obtained, for instance, excellent results in pig embryos which had been dropped in toto into chilled absolute alcohol at the slaughter house and later dissected in the laboratory for further fixation.

The material was kept for 24 to 48 hours in absolute alcohol in the refrigerator. From the alcohol the tissues were transferred to benzene at room temperature for 2 to 3 hours during which time the benzene was changed three times. The tissues were embedded in paraffin (Tissuemat, Fisher) of 56 to 58°C. melting point in an oven at 59°C. in the usual way. It is well to minimize the time during which the tissues are in the oven. A small vacuum oven, operated with a Pressovac pump, is useful in that it permits excellent embedding in less than one hour. The paraffin blocks were kept in the refrigerator until they were cut. The sections were left on the hot plate only long enough to permit flattening; the slides were then left to dry over night at room temperature.

Next the slides were freed of paraffin with three changes of xylene and were brought to water in the usual fashion without coating them with collodion. They were incubated for one and

[2] I wish here to acknowledge with gratitude the competent cooperation in various phases of this work of Miss JoAnne Sinden, Mrs. Virginia T. Wilson, Mrs. Doris McChesney, Dr. R. Weber and Dr. R. Frandson.

three-quarter hours at 37°C. in a solution prepared in the following manner:

One hundred cubic centimeters of a freshly made 2% solution of sodium glycerophosphate (52% α-, Eastman Kodak Co.) was added to 100 cm^3 of a freshly made 2% solution of sodium barbital. Next 200 cm^3 distilled water and 20 cm^3 of a 2% solution of anhydrous calcium chloride were added. The latter keeps indefinitely and need not be made up freshly each time. Finally 8 cm^3 of a 2% magnesium sulfate solution were measured carefully with a pipette and added to the other ingredients. The magnesium sulfate solution will keep indefinitely and need not be prepared freshly each time. The resulting 428 cm^3 were sufficient to fill several staining jars so that all slides of a complete series could be processed simultaneously.

After the slides had been incubated, the solution was discarded. The slides were rinsed in distilled water and next immersed 5 minutes in a 2% solution of cobaltous nitrate. This solution was saved and used over and over again. After treatment with cobaltous nitrate the slides were rinsed carefully in several changes of distilled water so that they did not carry any cobaltous nitrate into the next solution, which was an approximately 2% solution of light yellow ammonium sulfide. The sections remained 5 minutes in the ammonium sulfide in which they turned dark. They were then washed in running tap water for 10 to 15 minutes. After washing, the slides were counterstained with toluidin blue or carried directly through the usual series of alcohols and xylene. We passed the slides from the xylene to a jar with perchlorethylene in a well ventilated room (the vapors of perchlorethylene are injurious if inhaled for extended periods of time) and mounted the sections in clarite dissolved in perchlorethylene. This method has been recommended by Gomori for lipase preparations which will fade in xylene media. Mounting in clarite may not be necessary for alkaline phosphatase preparations, but we did not wish to take any chances with damar dissolved in xylene.

The sections used in this study were 15 μ thick. They show segments of blood vessels of varying length which may be measured for the quantitative determination of the vascularity of a given region as described by Craigie ('20, '24). If it is desired to reconstruct the vascular pattern from a number of sections various methods may be applied. The graphic reconstructions shown in figures 3 and 4 were prepared by drawing the outline of the area to be studied and its blood vessels of 10 consecutive sections with the help of a Leitz (Edinger) projector and superimposing them to obtain one combined drawing. The capillary fragments present in each section supplement each other so that the combined drawing of the capillary network looks much like that of a 150 μ thick section of brain tissue in which the blood vessels have been injected with India ink. There are several sources of inaccuracies, one of which is the distortion of the periphery of the projected field. However the resulting error can be kept relatively small by drawing only the center portion of the projected area. This method of reconstruction appears adequate to demonstrate the changes in the density and the pattern of the capillaries in different stages of the development of the central nervous system.

RESULTS

In a rat embryo (fig. 1) stained as described above the blood vessels of the brain and spinal cord appear black on a light background since their endothelial cells apparently contain a great deal more alkaline phosphatase than the brain tissue. In this selectivity the capillaries of the brain are unique; the blood vessels of other organs are not stained by the method described above (fig. 1). The significance of this biochemical difference between the blood vessels of the central nervous system and those of other organs cannot be discussed here, but seems interesting enough to deserve further investigation. In all parts of the central nervous system all capillaries stain uniformly (fig. 2). There are some areas where the nervous tissue also contains some alkaline phosphatase and consequently the contrast between blood vessels and nervous tissue

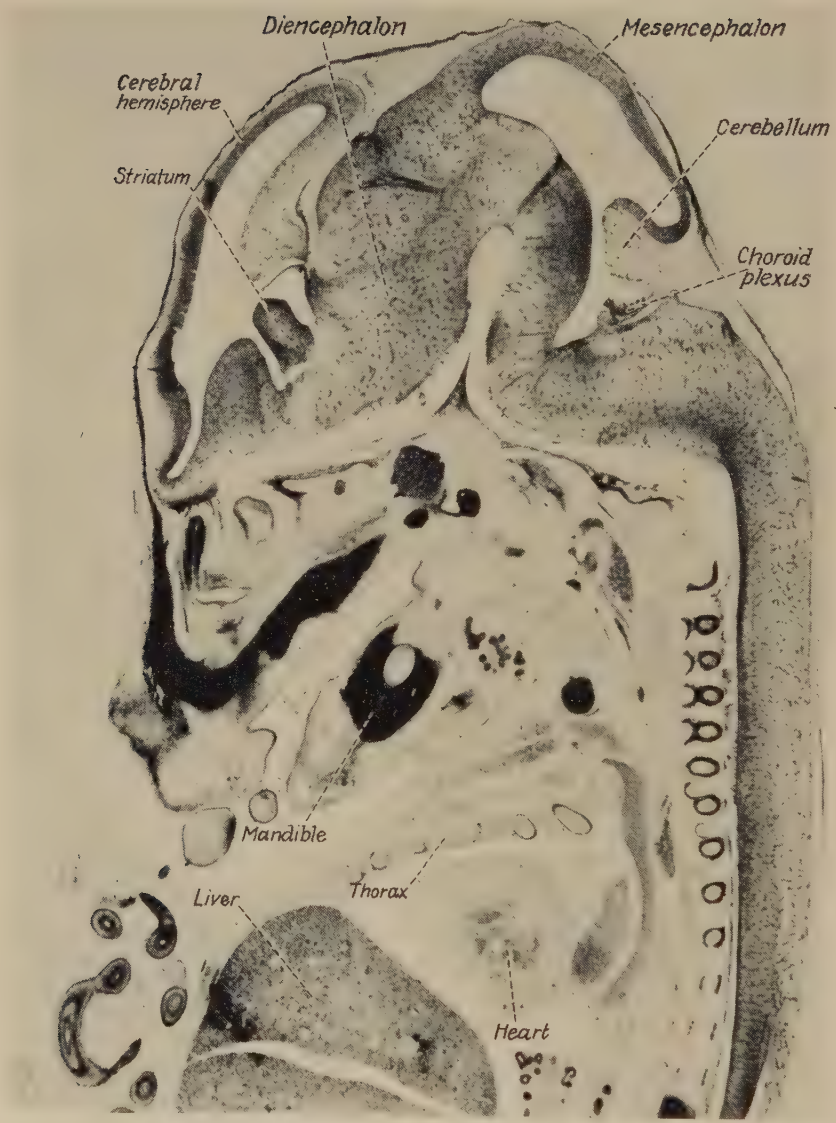

Fig. 1 Sagittal section through a rat embryo of 1.5 cm body length, 15 μ. Gomori's alkaline phosphatase method, photomicrograph. $\times$ 17. Note the ''staining'' of the blood vessels in the brain and spinal cord, and the absence of stained blood vessels in other organs. The developing bones show a strong alkaline phosphatase reaction.

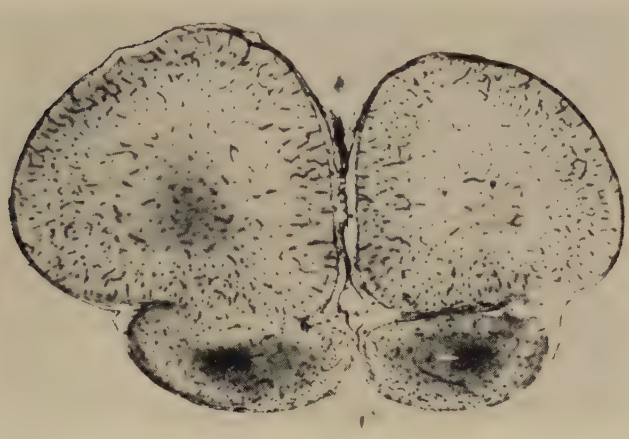

Fig. 2 Cross section through the anterior portion of the cerebral hemispheres of a rat fetus of 3.8 cm body length, 15 μ. Gomori's alkaline phosphatase method, photomicrograph. $\times$ 20. The blood vessels are selectively "stained."

Fig. 3 Graphic reconstruction of the capillary bed of the cornu ammonis and cortex of a rat embryo of 1.5 cm body length. The drawing was made from 10 consecutive sections of 15 μ each, and represents, therefore, the capillary bed of a corresponding section of 150 μ thickness (see text p. 323).

is less sharp. However, this does not interfere with the study of the architecture and density of the capillary bed throughout the central nervous system.

The cornu ammonis has been selected as an example (figs. 3 and 4) to illustrate the differences in capillary density in two different stages of development. A comparison of the vascular differentiation of the archicortex with that of the neocortex in the two stages of development confirms the observations of Craigie ('31) who found no precocious development of the blood supply of the cornu ammonis.

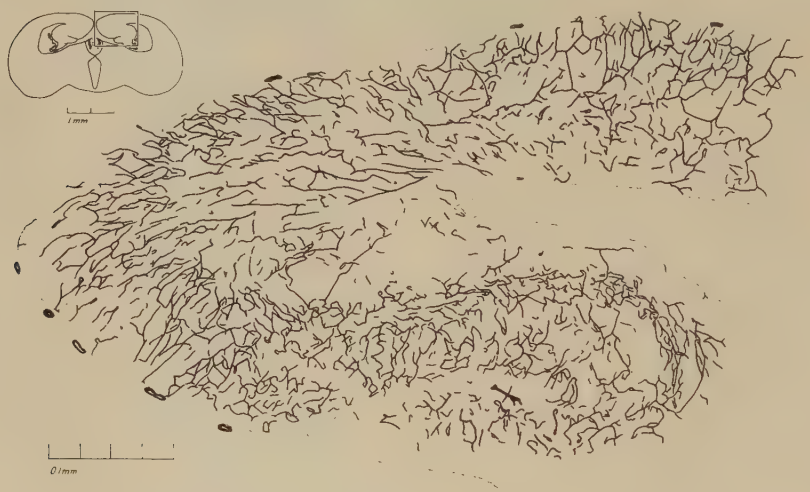

Fig. 4 Graphic reconstruction of the capillary bed of the cornu ammonis and cortex of a newborn rat of 4.6 cm body length. Technique as in figure 3. Note the density and differentiation of the capillary bed in comparison to that shown in figure 3.

SUMMARY

The use of Gomori's method for the demonstration of alkaline phosphatase in tissues is recommended for the study of the development of cerebral blood vessels. Since this method "stains" the endothelium of the blood vessels, the completeness with which the capillary bed of the fetal central nervous system may be demonstrated does not depend upon the patency of the vessels.

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ELECTRON MICROGRAPHS OF THE AMPHIASTER IN THE WHITEFISH BLASTULA (COREGONUS CLUPERFORMIS)[1, 2]

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NINE FIGURES

The architecture of the amphiaster has been and still remains an important problem in cytology. This is due, in part, to the fact that the elements of the aster and spindle cannot be clearly seen in most living cells. Accordingly, the fibrous appearance of the amphiaster often observed in fixed and stained preparations has been held as due to artifact induced by the fixation process. (For discussion of this point see Wilson, '37 and Fry-Wyssling, '48.) On the other hand, considerable evidence has recently accumulated to show that in certain living cells a typical fibrous appearance of the spindle and asters are present (Cleveland, '35). In addition, Schmidt ('36a, b), as well as others, have conclusively shown that both the components of the spindle and those of the aster, under polarized light, show birefringence in their long axis which means that they are composed of parallel oriented protein molecules. Other evidence derived from centrifuging and treatment with hypertonic solutions support the classical concept concerning the reality of the fibrous nature of the spindle and asters. Such evidence has led Schrader ('44) to

[1] We are indebted to Professor C. D. Janney of the Radiation Research Laboratory, State University of Iowa, for aid in the operation of the electron microscope.

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conclude that "The question of the reality of spindle fibers may be regarded as settled."

It is the purpose of this paper to present an enlarged view of the elements of the amphiaster as seen in electron micrographs. Since the material for this study had to be fixed and dried it obviously does not bear on the problem above mentioned, namely the possibility of extensive artifact induced upon fixation. We are accepting the view that what is seen here represents at least roughly the condition existing in the living cell. However, this must not be taken to infer that some degree of distortion has not occurred as will be discussed below.

MATERIAL AND METHODS

The material for this study consists of the developing blastula of the whitefish, kindly supplied us by the General Biological Supply House, Inc., Chicago, Illinois. The blastulae were fixed in a mixture of 90 cm^3 of 70% ethyl alcohol, 5 cm^3 of glacial acetic acid and 10 cm^3 of formaldehyde. They were subsequently embedded in 62°C. melting point "tissuemat" and sectioned at 0.2 to 0.5 μ by the aid of a no. 829 Spencer thin section adapter for the 820 Spencer rotary microtome. A special copper collar was fitted to the back of the metal microtome plug in such a way that ice water could be kept continually flowing through it. Thus, by conduction the mounted paraffin block containing the tissue could be kept at approximately 14°C. For sectioning, a Spencer blade holder equipped with a "Valet" razor blade was used. In front of the blade was attached a small glass well filled with an ice-chilled mixture of 45% ethyl alcohol and 2% glycerine. This adaptation was found to be very useful in keeping the sections from jamming on the blade and greatly facilitated their handling. The sections were spread on water at 45°C. and mounted on the conventional celloidin covered grids. When dry the paraffin was removed by two changes of xylol. No stains were added to the cells.

The electron microscope used was an R.C.A. Model E.M.U. equipped with an unbiased gun. Magnification of the electron micrographs is indicated by the micron scale mounted on each figure.

Terminology

As Schrader ('44) points out, the majority of the evidence indicates the presence of the following components associated with the mitotic figure: (1) "continuous fibers" connecting the two centers or poles; (2) "chromosomal fibers" which are connected with the kinetochore of the chromosomes and usually extend to the poles; (3) "interzonal fibers" extending between the separating chromosomes in anaphase and telophase; (4) center or pole, a morphologically distinguishable body toward which the spindle fibers and astral rays are oriented, which may or may not contain a centriole; (5) chromosomes, complex bodies, some or all of which may participate in the mitotic process.

The mitotic figures of dividing whitefish blastulae as seen through the light microscope are too well known to warrant a detailed description of them here. Excellent photographs of the amphiaster in this material may be found in catalog no. 17 of the General Biological Supply House, Inc., Chicago, Illinois, or in Sharp ('34). We chose to study the dividing whitefish blastula because of its striking asters and well developed spindle. However, no centriole is visible in either of the asters. The fixed cytoplasm gives the appearance of a fibrous network.

Electron micrographs of dividing cells

A. Spindle. It should be pointed out that electron micrographs of non-dividing cell protoplasm show it to be a relatively unoriented fibrous network. This condition probably represents the coagulated "structural proteins" within the cell. Presumably certain of the "mobile proteins" as well as the lipid containing components have for the most part been removed by the method of preparation.

In figure 1 is represented what appears to be a metaphase amphiaster. However, a distinct doubling of the chromosomes is not evident. An enlarged view of a portion of the equatorial plate of this cell is illustrated in figure 3. Here relatively large distinct oriented fibers are clearly seen. The fibers vary in size from 1,000 to 3,000 Å and appear continuous. Smaller interconnecting fibers are also evident in many parts of the spindle. Such a condition is in harmony with the view that the spindle consists of a jelled region. In these figures it will be observed that it is not possible to distinguish clearly (by a study of the morphology alone) between the various types of spindle fibers, i.e., "chromosomal" and "continuous." Neither is it possible to observe the kinetochore of the chromosomes.

The identity of a short portion of a "chromosomal fiber" seems definite in figure 4. However, here as in the other figures, no details of its attachment to the chromosome can be made out. Unpublished data on other types of dividing cells show promise of possible analysis of this point with the electron microscope. It is interesting to note that the spindle fibers do not vary greatly in size and are clearly oriented in the direction of the poles. Such a condition argues for the view that both the "chromosomal" and "continuous" fibers are structurally fundamentally the same. That is, both are derived from the fibrous micellar hyaloplasm by an orientation of its ultrastructure. How certain of these fibers ("chromosomal") become attached to the chromosomes is not clear from these figures.

B. Anaphase. Figures 2 and 5 show stages in which the chromosomes are nearing the astral center. Typical large fibrous oriented spindle elements are present. In fact, figuratively speaking this condition of the fibers looks like an elongated "brush heap," to use the expression of Seifriz ('36). Between the larger orienting fibers may be seen smaller interconnecting strands. Figure 5 especially demonstrates how the spindle elements lose their orientation at the periphery of the central portion of the aster. Many of the

spindle fibers appear beaded, or banded, a condition which is possibly due to irregular contractions at the time of fixation (fig. 3). Efforts thus far have failed to disclose a regular periodic banding of the fibers of the same relative order of spacing like that demonstrated for collagenous fibers. However, further work may eventually demonstrate their presence.

In figure 2 a more advanced stage of mitosis is present. Here certain of the chromosomes appear to be breaking down as is evidenced by the lighter and darker areas present within them. The spindle elements show evidence of degeneration as noted by their less regular alignment, robust appearance and by the apparent interruption of certain of them. Certainly by this time a solation of the spindle is occurring and here may be evidence as above described of the morphological changes accompanying this process.

Asters. It is relatively easy to identify the central region of the asters on the fluorescent screen because of their relatively dense reticular sponge-like structure (figs. 5, 6). This dense reticulum differs from any other region of the cell. In well developed asters the rays appear structurally similar to those of the spindle elements except that they vary more in diameter and in some cases appear to be discontinuous or broken. The appearance of a small portion of an aster (two rays) extending from their center or origin to their attachment at the cell membrane is illustrated in figures 6, 7, 8, 9. In this composite picture figures 6 and 7 do not exactly overlap in all regions. However, as the rays emerge from their astral focal point they are oriented for the most part toward the cell membrane (bottom of fig. 9). They are not smooth but irregularly banded, a condition possibly due to contraction induced upon fixation or drying. The interconnecting fibers are more numerous here than they are where the astral rays approach the surface of the cell. More centrifugally the astral rays clearly appear more widely separated and to be composed of one or more fibers (figs. 8, 9). In the cortex region they still remain relatively widely separated and seem to be

associated with more irregular dense masses of coagulated material. The rays are definitely attached to the membrane of the cell (bottom of fig. 9).

How nearly these figures of the aster represent the condition in the living cell cannot be stated. Some degree of artifact undoubtedly exists but it should be remembered that both Heilbrunn ('20) and Monné ('44) have presented good evidence that the astral rays are fibrous.

It is conceivable, as pointed out by Chambers ('24), that the clear spaces between the fibers here illustrated might represent regions where oriented cytoplasmic flow could occur.

DISCUSSION

It is obvious that the electron micrographs presented here of the amphiaster substantiate the view that it is composed of fibers, a condition long ago described by many cytologists. It was not possible here to differentiate on a structural basis a difference between the "chromosomal" and "continuous" fibers. Only in those cases where the "chromosomal fibers" were observed to be attached to the chromosomes could their identity be determined with certainty. However, unpublished data indicate that the identity of the various types of spindle fibers may be easily established in other types of dividing cells. In fact, the chromosomal fibers of certain crayfish cells appear to be composed of smaller fibrous elements. However, we are inclined, for the present, to view the elements of the amphiaster in the whitefish blastula, i.e., "continuous fibers," "chromosomal fibers," and possibly astral rays, as morphologically the same except for size. In other words, they all appear to be derived from the basic ground cytoplasm which is composed of an anastomosing protein meshwork which becomes oriented under the influence of the astral centers. This condition is undoubtedly associated with the change in viscosity of the cell that is known to occur at this time.

In other words, the work here reported seems completely in accord with the view expressed by F. O. Schmitt ('39, '40) that "The results of the polarization and x-ray optical analy-

sis are in agreement with the view that animal fibers, whether in large compact bundles (muscle, tendon), or microscopic and intracellular (chromosomes, spindle and astral fibers) are constructed of an anastomosing meshwork of sub-microscopic fibrous particles or micelles oriented with long axes parallel to the fiber axis . . . Data on extensility and viscosity require that the particles be interlinked by covalent strands such as compose the particles themselves, although the greater fraction of the strands are longitudinally oriented.'' Monné ('44) found by using polarized light on the living sea urchin egg that it, too, was composed of fibrils made up of bundles of polypeptide chains which join in the form of a texture. It is known that fibers such as collagen when treated with acid, or with pepsin, fragment longitudinally into fine filaments which retain their periodicity in the axial direction. They may even be made to pass through filter paper, and upon neutralization of the medium under certain conditions will reorganize themselves into new fibrillae with a periodic axial structure (De Robertis, Nowinski and Saez, '48). Even some proteins in globular form such as insulin or actin, a protein extracted from muscle, may under suitable conditions be reversibly transformed into a fibrous state. It is conceivable as pointed out by Schmitt ('39), Lazarow ('43) and others that some such mechanism may be operating in the formation and destruction of the asters and spindle fibers. Such a condition could conceivably be controlled by special enzyme systems.

We were not successful in observing periodic banding in either the astral or spindle fibers like that so beautifully demonstrated for collagen (Schmitt and Gross, '48). However, further work using electron strains, shadow casting and mitotic poisons may eventually demonstrate their presence.

It seems unnecessary to discuss here the numerous papers dealing with the spindle fibers since, as previously mentioned, an excellent review of this subject has recently been published by Schrader ('44). We are well aware that considerable variation may exist in spindle elements in different types of cells.

Accordingly we wish to make no claim that what we have reported here for dividing whitefish blastula will necessarily hold for all types of amphiasters.

SUMMARY

Electron micrographs of dividing whitefish blastulae have been made. Certain adaptations for thin sectioning such as a method for cooling the block and for removing the sections from the knife have been described.

The amphiaster elements such as "chromosomal fibers," "continuous fibers," and astral fibers have been observed, oriented in the same manner as described for them under the light microscope. In the whitefish the spindle elements are nearly the same size and seem to be constructed in the same way. In other words, it was difficult to identify one type spindle fiber from the other except in those cases where their attachments could be established. They often showed a gross but no periodic banding of their utrastructure. Inner connecting strands, that is, smaller fibers extending between the more grossly oriented spindle elements, were observed.

The astral rays appear fibrous and oriented from the dense reticular center toward the periphery of the cell. They may or may not appear continuous, probably depending on whether or not they have been broken by the technique of preparation. Numerous smaller unoriented fibers extend between the astral rays.

The ground cytoplasm has a sponge-like appearance. This is apparently the protein component of the cytoplasm commonly referred to in the literature as "structural" or "framework cytoplasm."

From the existing literature on fibrous proteins it is conjectured that the asters and spindle elements are derived from the structural cytoplasmic framework by orientation of its ultrastructure much in the same way that others have demonstrated for the organization and disorganization of certain other noncellular fibrous protein elements.

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PLATE 1

EXPLANATION OF FIGURES

1 Metaphase showing chromosomes, ''chromosomal fibers,'' ''continuous fibers'' and asters. Scale: 10 μ.

2 Late anaphase. Here the elements of the spindle show evidence of disorganization by their less sharp orientation toward the poles, apparent discontinuity of some of them and in general by their less robust appearance as compared to figure 1. Scale: 2 μ.

1

2

PLATE 2

EXPLANATION OF FIGURE

3 Enlarged view of portion of metaphase plate of figure 1. The dense body in center is a chromosome, about which are seen the spindle fibers. Scale: 1 μ.

1μ
3

PLATE 3

EXPLANATION OF FIGURES

4 Chromosome with fiber attached.

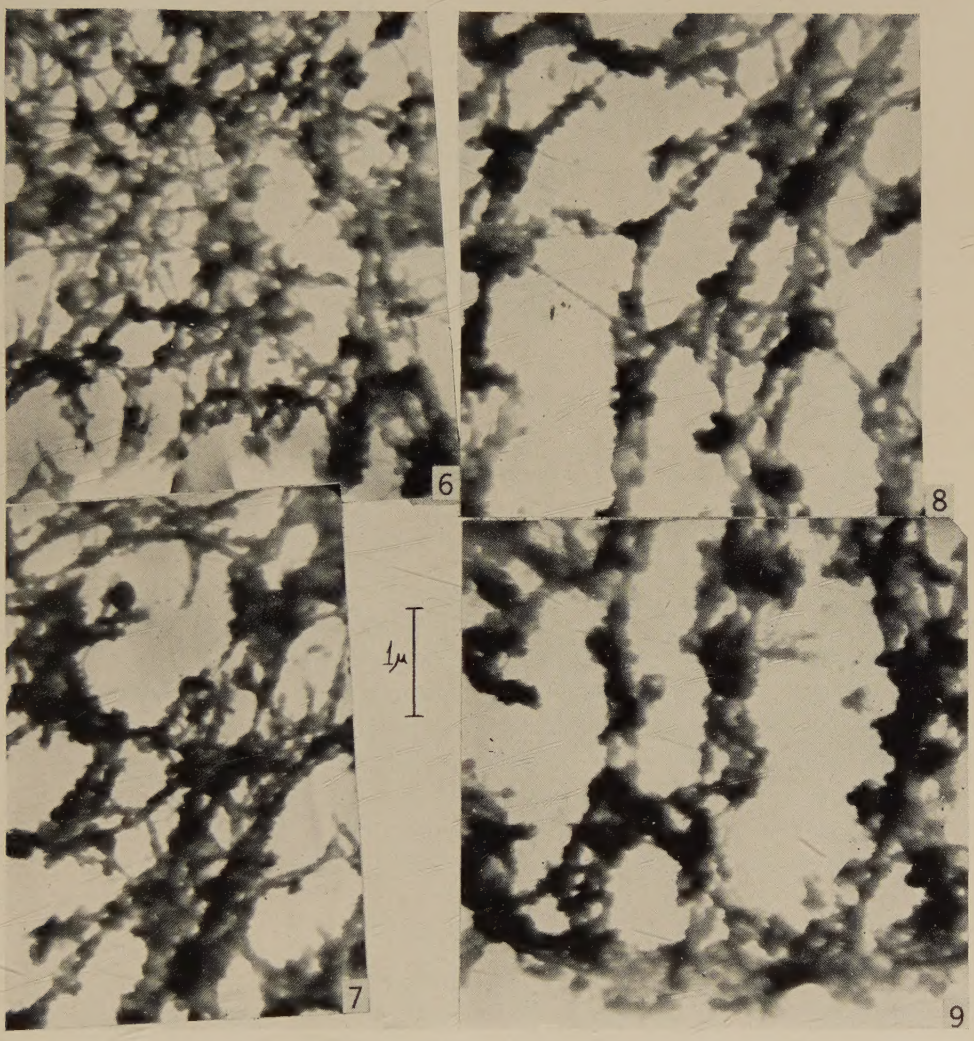
5 Anaphase showing spindle fibers and center of aster. Here the difference in organization of the astral center and rays may be seen clearly. Scale: 1 μ.

1 μ
4
1μ
5

PLATE 4

EXPLANATION OF FIGURES

6, 7, 8 and 9 Consecutive figures of a portion of aster showing its organization from center (fig. 6) to its attachment to cell membrane at bottom of figure 9. Scale: 1 μ.


6
8
1μ
7
9